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UNIVERSITY OF ALBERTA

Effect of Environmental Stress on Planktonic and Biofilm Cells

BY

Janice L. Strap

A thesis submitted to the Faculty of Graduate Studies and Research in partial fullfillment of the requirements for the degree of Master of Science.

DEPARTMENT OF MICROBIOLOGY

Edmonton, Alberta
Fall, 1993

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Janice L Strap
(student's signature)

Student's permanent address:

15824 78 Street
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"There is only one thing which will really train the human mind, and that is the voluntary use of the mind by the man himself. You may aid him, you may guide him, you may suggest to him, and, above all you may inspire him; but the only thing worth having is that which he gets by his own exertions: and what he gets is proportionate to the effort he puts into it."

-- A. Lawrence Lowell --

"God gives every bird its food, but he does not throw it into the nest."

-- J.G. Holland --

"Nonne vides etiam guttas in saxa cadentis
Umoris longo in spatio pertundere saxa?"

-- Lucretius --

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Brenda Leskiw

Dr. B. K. Leskiw - Supervisor

Dr. S. E. Jensen

Dr. M. A. Pickard

Dr. M. Peppler

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This thesis is dedicated to Peter, Phyllis, Shirley and Andrew. Without their love and support, this work would not have come to completion.

ABSTRACT

Colonization of medical devices (such as urinary catheters and cardiac pacemakers) by bacteria forming biofilms is a significant problem associated with invasive therapeutic techniques. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are common pathogens involved in such device-related infections.

The ability of *S. aureus* to colonize lengths of silicone tubing was investigated by cultivating the organism under iron-limitation in a chemostat. Biofilm cell numbers increased exponentially for the first six days, reaching a maximum density after cultivation for 11 days. The susceptibility of young and old biofilm cells as well as their associated planktonic cells was investigated by exposure to tobramycin, cephalexin, and a combination of these antibiotics. Planktonic cells exposed to antibiotics external to the chemostat system were completely killed by exposure to a combination of 5 μg/mL tobramycin plus 100 μg/mL cephalexin. Biofilm cells were more resistant and were not eliminated at this concentration. The resistance of the biofilm culture to the antibiotic concentrations used appeared to increase with increasing age. Administration of antibiotic directly into the chemostat at a loading dose of 70 μg/mL tobramycin and 500 μg/mL cephalexin and maintenance doses of 62.5 μg/mL tobramycin and 250 μg/mL cephalexin resulted in complete killing of both young biofilm and planktonic cultures. Old biofilm cultures and their associated planktonic cells showed a reduction in viability but were not eliminated with these concentrations of antibiotics.

P. aeruginosa, an important opportunistic biofilm-forming pathogen, was cultivated under iron-limitation in chemostat cultures under varying dilution rates to determine the effect of dilution rate on the expression of iron-

regulated outer-membrane proteins of biofilm cells. The population of planktonic cells decreased when the dilution rate was increased. In contrast, the biofilm population increased when the dilution rate was increased. As expected, expression of the iron-regulated outer-membrane proteins was only induced at the lower dilution rates.

The age of mucoid *P. aeruginosa* biofilms affected their susceptibility to tobramycin and piperacillin. Planktonic and young biofilm cells were eradicated after 6 doses of 5 μg/mL tobramycin and 500 μg/mL piperacillin. Eradication of old biofilm cells was not achieved with these concentrations of antibiotics.

Alginate, the exopolysaccharide of mucoid *P. aeruginosa* is thought to be an important virulence factor, and is also believed to aid in the formation of biofilm. Transcriptional activity of *algD*, the structural gene for a key enzyme in the biosynthesis of alginate, was investigated under a variety of environmental conditions using a strain containing a chromosomally located transcriptional fusion of *algD* and the reporter gene *xylE*. Growth on solid media appeared to show elevated levels of *algD* promoter activity in response to high osmolarity. Experiments performed in batch culture were inconclusive regarding the influence of osmolarity on *algD* transcription.

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ABBREVIATIONS

ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
Cb	Carbenicillin
CDO	Catechol 2,3 dioxygenase
CF	Cystic Fibrosis
CFU	Colony Forming Units
D	Dilution rate (hr^{-1})
IROMPs	Iron-regulated outer-membrane proteins
LB	Luria Bertani medium
MIC	Minimum Inhibitory Concentration
min	minute
NaCl	sodium chloride
OM	Outer membrane
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered Saline
PIA	*Pseudomonas* isolation agar
rpm	revolutions per minute
Sarcosyl	N-lauryl sarcosinate
SDS	Sodium dodecyl sulfate
TSB	Tryptic soy broth

I. INTRODUCTION

A limiting factor for the growth of heterotrophic microbes in nature is the low concentration of usable organic and inorganic matter (Characklis and Marshall, 1990; Costerton *et al.*, 1987). This creates the problem of how to acquire sufficient nutrients for survival and reproduction. Bacteria have had to acquire certain adaptive strategies for the procurement of essential nutrients by developing ingenious uptake systems as well as methods of attachment to solid surfaces. Attachment to surfaces in dilute milieus has definite advantages for bacterial cells: adhesion allows the rapid absorption of nutrients as they flow past because the glycocalyx produced by the adherent cells functions as an ion-exchange resin to trap nutrients (Costerton *et al.*, 1987; Costerton and Irvin, 1981; Van Loosdrecht *et al.* 1990; Christensen *et al.*, 1989). Microbial mechanisms of attachment involve the physicochemical interaction of cellular surface macromolecules with an inert surface (Characklis and Marshall, 1990). Adherence to such surfaces results in the formation of biofilm. At this point, the distinction between planktonic and biofilm cells must be emphasized. Planktonic cells are free-living, while biofilm cells are sessile (attached to a surface). Fimbriae, flagella, lipopolysaccharide and extracellular adhesive polymers (glycocalyx) are believed to take part in the initial adherence processes involved in biofilm formation (Costerton and Irvin, 1987; Van Loosdrecht *et al.*, 1990).

Biofilms can develop on any material placed in a biologically active liquid (Costerton *et al.*, 1981; Dudman, 1977). They can be beneficial or detrimental. Examples where biofilms are of benefit include: (1) water quality as biofilms influence dissolved oxygen content and serve as a sink for toxic

and/or hazardous materials (Bryers and Characklis, 1990; Characklis and Marshall, 1990), and (2) reactors where biofilms are used for commercial fermentation processes such as the manufacture of vinegar (Characklis and Marshall, 1990). As stated above, biofilms can also be detrimental. Notable examples include the involvement of biofilms in colonization of medical devices, in corrosion and in biodeterioration (Costerton *et al.*, 1987; Little *et al.*, 1990).

Modern medical care is often invasive, and a large number of plastic and metal medical devices have been developed for implantation into patients as effective therapeutic interventions including urinary catheters, artificial joints and cardiac pacemakers (Jacques *et al.*, 1987). Electron microscopic examinations have revealed that pathogenic bacteria establish life-threatening biofilms when they colonize medical devices (Daifuku and Stamm, 1984; Kunin and Steele, 1985; Holmes and Evans, 1986; Jacques *et al.*, 1987; Dickinson and Bisno, 1989; Vas, 1989; Marrie and Costerton, 1990; Nickel *et al.*, 1985a). Epidemiological studies have revealed that organisms found in the normal skin flora, such as *Staphylococcus epidermidis,* are commonly associated with device-related infections (Daifuku and Stamm, 1984; Kunin and Steele, 1985; Holmes and Evans, 1986; Jacques *et al.*, 1987; Dickinson and Bisno, 1989; Vas, 1989; Marrie and Costerton, 1990). Coagulase-negative Staphylococci such as *S. epidermidis* adhere readily to devices inserted through the skin or implanted beneath it. On the other hand, infections caused by Gram-negative bacteria such as *Pseudomonas aeruginosa* are often found to be associated with prostheses that are partially or fully exposed to the environment (Daifuku and Stamm, 1984; Kunin and Steele, 1985; Holmes and Evans, 1986; Jacques *et al.*, 1987; Dickinson and Bisno, 1989; Vas, 1989; Marrie and Costerton,

1982; 1990). Greater than 80% of peritonitis in patients receiving Tenckhoff catheters is caused by *Staphylococcus aureus* and *S. epidermidis* (Vas, 1989). Bacterial biofilms on the surface of biomaterials often do not cause overt infection or detectable inflammation (Costerton *et al.,* 1987), however these biofilms are able to give rise to disseminating planktonic cells which can trigger clinical symptoms (Costerton *et al.,* 1987).

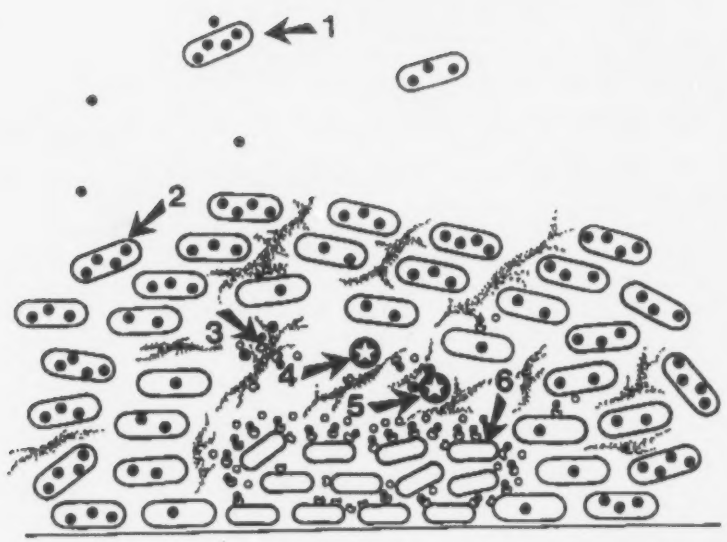
The bacterial glycocalyx can be defined as any polysaccharide-containing component outside the cell wall (Costerton and Irvin, 1981). It is a hydrated polymeric matrix, composed of 99% water (Sutherland, 1979). This polyanionic matrix influences the access of molecules and ions (Costerton *et al.,*1983) to the cell wall and cytoplasmic membrane. Exopolysaccharide formation is favored by the presence of an excess of carbohydrate and a limitation of other nutrients such as nitrogen, phosphorus and sulfur (Sutherland, 1977). Physical conditions (growth on solids versus liquids) have also been shown to influence the amount and the chemical composition of the exopolysaccharide produced (Fletcher and Floodgate, 1973; Sutherland, 1977).

Since *S. aureus* and *P. aeruginosa* are major pathogens in medical device-associated infections, adherance mechanisms employed by these pathogens have been the subject of intensive investigations. As already stated, the process of adhesion involves complex electrostatic and hydrophobic interactions between the surface components of the cells and the device (Characklis and Marshall, 1990; Van Loosdrecht *et al.,* 1990; Christensen *et al.,* 1989). Newly adherent organisms begin to divide and, as time elapses, microcolonies are formed within a glycocalyx matrix. Micocolonies coalesce to form biofilms as the size and number of adherent microcolonies increase (Costerton *et al.,* 1987; Van Loosdrecht *et al.,* 1990). Adherent microcolonies

are protected from antibacterial agents including antibiotics and phagocytes (Costerton and Irvin, 1981) enabling them to persist.

Planktonic and biofilm cells are known to coexist at the site of infection (Costerton *et al.*, 1987). The physiology of bacteria in biofilms is complex and very different from those of planktonic cells. The physiology of bacteria in biofilms is likely to be influenced by the sites in which each individual biofilm cell is located in the multilayers of cells which compose the biofilm (Caldwell and Lawrence, 1986; Costerton *et al.*, 1987; Lawrence *et al.*, 1987; Van Loosdrecht *et al.*, 1990). Cells located on the surface of the biofilm may have easier access to nutrients and have fewer problems with the clearance of metabolic waste-products. Cells embedded deep within the glycocalyx matrix are likely to be less metabolically active due to poor access to essential nutrients, and in addition they must cope with waste-metabolite accumulation in their milieu. Since it is already well established that the physiology of the bacterial cell can profoundly influence the sensitivity of the cell to antibiotics (Anwar *et al.*, 1990; Costerton *et al.*, 1987), it follows that the mechanisms of resistance of biofilm bacteria to antibiotics would probably be very complex, due to the elaborate nature of biofilm physiology. It has been proposed that biofilm cells embedded in the glycocalyx matrix may have different degrees of susceptibility to antibiotics, depending on the sites where the individual cells are located within the multiple layers of cells forming the biofilm (Plate 1). For

PLATE 1. A proposed model for describing the complex nature of the resistance of bacteria in biofilm to antibiotics. Step 1: Planktonic cells around the infected catheter. These cells are large in size, and the cell membrane is permeable to nutrients and antibiotics. Step 2: Surface biofilm cells. These cells resemble the planktonic cells. They are large in size, and the cell membrane is permeable to nutrients and antibiotics. Step 3: Binding of antibiotic molecules to the exopolysaccharide (glycocalyx) produced by biofilm cells. Step 4: Immobilization of antibiotic-degrading enzymes on the glycocalyx matrix. Step 5: Inactivation of antibiotic molecules by antibiotic-degrading enzymes immobilized on glycocalyx matrix. Step 6: Embedded biofilm cells. These cells are dormant due to an inaccessibility of essential nutrients. They are smaller in size, and the cell membrane has been physiologically adjusted to be less permeable to antibiotic molecules. The events described in steps 1 to 5 reduce the rate and the number of antibiotic molecules that reach the embedded biofilm cells. These cells will therefore have sufficient time to switch on the production of antibiotic-degrading enzymes to facilitate the inactivation of antibiotic molecules. The embedded biofilm cells in old biofilms survive the onslaught of antibiotic molecules. The stars represent antibiotic-degrading enzymes.
(A similar figure appeared in Anwar *et al.*, 1992c).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example, cells deep within the glycocalyx matrix are likely to be resistant whereas those nearer the surface are likely to be more susceptible. This is because embedded cells are protected not only by their covering of glycocalyx but also by overlying cells. These two factors combined comprise an effective physical barrier against antibiotics, host defenses, and fluctuations in the surrounding environment (Gilbert *et al.*, 1990).

ß-Lactam antibiotics in combination with aminoglycosides are commonly used for the treatment of device-associated infections (Anwar and Costerton, 1990; Eliopoulous, 1986; and Klastersky *et al.*, 1972). ß-Lactams like penicillin and cephalosporin enhance the permeability of the bacterial cell and thereby facilitate the entry of the aminoglycoside (La Du *et al.*, 1971). ß-Lactam antibiotics inhibit peptidoglycan synthesis by targeting the transpeptidation reaction involved in the cross-linking step of peptidoglycan biosynthesis. This class of antibiotics has a relatively low toxicity (La Du *et al.*, 1971). Aminoglycoside antibiotics inhibit bacterial protein synthesis by attaching to and inhibiting function of the 30S subunit of the bacterial ribosome (La Du *et al.*, 1971). In an *in vitro* system designed to assess antibiotic eradication of biofilm, it has been shown that both ß-lactam and aminoglycoside antibiotic molecules can penetrate newly formed biofilms (Anwar and Costerton, 1990; Anwar *et al.*, 1989a; 1990; Nichols *et al.*, 1989). In addition, biofilm cells that are sloughed off from the surface of the substratum have also been shown to be sensitive to antibiotics, presumably because by becoming planktonic they are no longer protected by the biofilm mode of growth (Haag *et al.*, 1986).

When cells involved in an infection are exposed to antibiotics, the planktonic and surface biofilm cells may be quickly inactivated because they

are actively growing and therefore susceptible to antibiotics. The antibiotic molecules that do not interact with the planktonic and surface biofilm cells, and are unaffected by the action of degradative enzymes, presumably continue their journey to the embedded biofilm cells (as stated above some classes of antibiotics have been shown to penetrate new biofilms). The glycocalyx produced by biofilm cells is negatively charged and is known to function as an ion-exchange resin (Costerton *et al.*, 1987) which impedes antibiotic penetration (Costerton *et al.*, 1987; Nickel *et al.*, 1985b; Slack and Nichols, 1981; 1982), and it was found that the exopolysaccharide cannot be penetrated by certain antibiotics in sufficiently high concentrations to affect the embedded bacterial cells (Isenberg, 1988). Antibiotic degrading enzymes such as ß-lactamases may also be immobilized on the glycocalyx matrix so that the incoming antibiotic molecules may be inactivated (Giwercman *et al.*, 1992). Furthermore, the embedded biofilm cells may not be actively engaged in cell division and may be smaller in size (Costerton, 1988). Slow-growing cells are generally less susceptible to antibiotics (Brown and Williams, 1985; Eng *et al.*, 1991; Gilbert *et al.*, 1990). In addition, the ability of antibiotic molecules to cross the outer membrane of the old Gram-negative biofilm cells may be reduced significantly as a result of the alteration of the composition of the outer membrane (Anwar *et al.*, 1990; Costerton *et al.*, 1987; Nichols *et al.*, 1989).

In addition to the biofilm mode of growth, iron availability also influences the physiology of bacterial cells growing *in vivo* (Costerton, 1988). Although iron is an essential nutrient for the growth of most microorganisms and is required for a number of metabolic activities, it is not freely available to bacteria growing within the body. The concentration of free iron in body fluid

is reported to be approximately 10^{-16}M (Griffiths, 1983; Neilands, 1981; 1982; Weinberg, 1978; 1984). The low free iron concentration is due to endogenous chelating compounds such as transferrin (found in serum and lymph) and lactoferrin (found in secretions and milk) which have high affinities for iron (Briat, 1992; Brown and Williams, 1985; Griffiths, 1983). The restriction of available iron is an important nonspecific host defense against microorganisms (Anwar *et al.*, 1984; Brown *et al.*, 1984; Griffiths, 1983). In order for bacteria to survive in biological fluids they have had to develop ways to sequester iron from the low iron environment. Iron deprivation, inherent in growth within infected tissues, profoundly changes the cell wall protein composition of Gram-negative bacteria (Brown and Williams, 1985). The cultivation of microorganisms under iron deprivation induces the production and secretion of microbial iron chelators (Griffiths, 1983; Neilands; 1981; 1974), and the expression of iron-regulated outer-membrane proteins (IROMPs) which function as receptors for iron-siderophore complexes involved in iron uptake accross the outer membrane (OM) (Anwar *et al.*, 1984; Brown *et al.*, 1984; Chart and Griffiths, 1985; Griffiths, 1983; Lam *et al.*, 1984; Lodge *et al.*, 1986; Shand *et al.*, 1985; 1988; Ward *et al.*, 1988). Secretion of siderophores, which have a high affinity for ferric iron, enables microbial pathogens to grow within their host by securing a supply of iron (Archibald and DeVoe, 1980; Briat, 1992; Griffiths, 1983; Herrington and Sparling, 1985; McKenna *et al.*, 1988). Iron-regulated outer-membrane proteins are expressed by pathogenic bacteria *in vivo*, and are recognized by antibodies present in convalescent sera (Shand *et al.*, 1985; Ward *et al.*, 1988). In this way, iron acquisition by invading pathogens is inhibited.

In addition to being associated with the colonization of medical devices, *S. aureus* and *P. aeruginosa* also colonize the cystic fibrosis (CF) lung. *S. aureus* is a coagulase-positive pathogen responsible for many severe infections and is often recovered from the respiratory tract of chronically infected CF patients (Gilligan, 1991; Govan and Nelson, 1992). Adherence of *S. aureus* to respiratory epithelium involves the cell wall component, teichoic acid, and the extracellular layer of slime associated with these organisms. The slime produced is biochemically distinct from exopolysaccharides produced by *P. aeruginosa* (Gilligan, 1991). Other virulence factors are produced by *S. aureus* including hemolysins, catalase, coagulase and several exotoxins (Gilligan, 1991) which compound problems associated with the biofilm mode of growth (in particular, the inherent resistance to antibacterial agents). Tissue damage caused by *S. aureus* is thought to predispose the lung of cystic fibrosis patients to pseudomonal colonization (May *et al.*, 1991; Govan and Nelson, 1992), the exact mechanism for this remains unclear (Gilligan, 1991; Govan and Nelson, 1992).

P. aeruginosa is nearly ubiquitous in natural environments and is usually innocuous. It is an opportunistic pathogen which produces a number of unique virulence factors that make it particularly adept at infecting specific host tissues. Extracellular toxins, proteases, hemolysins, and exopolysaccharides are a few types of virulence factors that have been implicated in the pathogenicity of *P. aeruginosa* (Govan, 1988; Govan and Harris, 1986). *P. aeruginosa* can cause severe and life-threatening infections in immunocompromized hosts such as burn patients, patients suffering from respiratory disease, cancer chemotherapy patients, and children and young adults with CF (Govan and Harris, 1986). In many instances, this organism

grows within sheltered biofilm communities held together by glycocalyx which protects it from antibiotics and the host's immune system (Costerton *et al.*, 1987; May *et al.*, 1991).

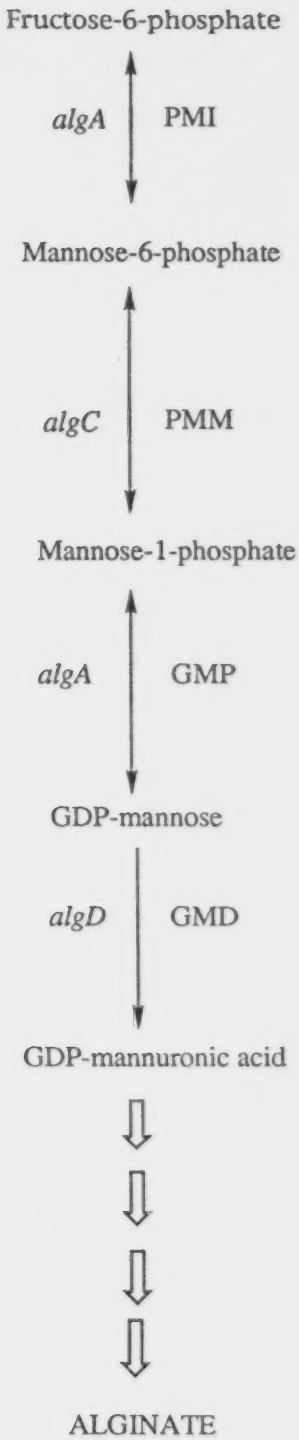
The mucoid phenotype of *P. aeruginosa*, which has been shown to result from increased levels of alginate biosynthesis, is an important virulence factor in cystic fibrosis (Govan and Harris, 1986). The cystic fibrosis lung has elevated levels of sodium chloride. Respiratory tract fluid of CF patients contains high levels of Na^+ and Cl^- ions (90 and 80 mM respectively) (Kilbourne, 1978). Increased concentrations of this osmolyte have been reported to increase the transcription (Deretic *et al.*, 1990) of the *algD* gene, which encodes GDP-mannose dehydrogenase. This gene has been shown to be essential for alginate synthesis (Deretic *et al.*, 1990).

Alginate, a negatively charged linear co-polymer of O-acetylated ß(1-4) linked D-mannuronic acid and variable amounts of its C-5 epimer L-guluronic acid (Evans and Linker, 1973), is of industrial and medical importance. It is synthesized as an exopolysaccharide by mucoid strains of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients (DeVault *et al.*, 1989; Berry *et al.*, 1989). Alginate production is thought to be a mechanism by which *P. aeruginosa* resists dehydration in the CF-affected lung (Govan and Harris, 1986) because of the highly hydrated nature of alginate (Dudman, 1977). Mucoid forms of *P. aeruginosa* are rarely encountered in nature and their appearance is restricted to a very limited ecological niche present in CF lungs (Dogget, 1969; Govan and Harris, 1986). Mucoid strains are recalcitrant even to potent anti-pseudomonal antibiotics (Govan and Harris, 1986). *P. aeruginosa* replaces the initial pathogens, *Staphylococcus aureus* and *Haemophilus influenzae*, to become the sole pathogen, shifting

characteristically into the mucoid phenotype during the terminal stages of the disease (Burns and May, 1968; Govan and Nelson, 1992; Williams and Govan 1973; Diaz *et al.*, 1973). Such isolates spontaneously produce non-mucoid variants *in vitro* (Govan, 1975).

The alginate biosynthetic pathway is shown in Figure 1. The first alginate precursor for the *P. aeruginosa* biosynthetic pathway is fructose 6-phosphate and appears to be recruited from the carbohydrate pool via the Entner-Doudoroff pathway (Banerjee *et al.*, 1983; Carlson and Matthews, 1964; Lynn and Sokatch, 1984) and fructose 1,6-bisphosphate aldolase (Banerjee *et al.*, 1985). Piggott *et al.* (1981) first demonstrated the presence of the alginate biosynthetic enzymes phosphomannose isomerase (PMI), GDP-mannose pyrophosphorylase (GMP), and GDP-mannose dehydrogenase (GMD). Phosphomannomutase (PMM), another alginate biosynthetic enzyme was detected by Padgett and Phibbs (1986). The enzymatic activities of these alginate biosynthetic enzymes are low, even in the most highly mucoid strains of *P. aeruginosa* (May *et al.*, 1991, Padgett and Phibbs, 1986, Piggott *et al.*, 1981, Martins and Sá-Correia, 1991). The method by which GDP-mannuronic acid and GDP-guluronic acid are incorporated into *P. aeruginosa* alginate is not fully understood (Chitnis, 1990; May *et al.*, 1991), however, the production of bacterial exopolysaccharides occurs at the level of the cytoplasmic membrane and is thought to involve polyprenol carrier lipids (Roychoudhury *et al.*, 1989; Sutherland, 1977).

FIGURE 1. Biosynthetic pathway of alginate in *Pseudomonas aeruginosa*. Abbreviations: PMI, phosphomannose isomerase; PMM, phosphomannose mutase; GMP, GDP-mannose pyrophosphorylase; GMD, GDP-mannose dehydrogenase. The genes encoding the enzymes are shown. Open arrows indicate polymerization, epimerization, acetylation, and export.


Fructose-6-phosphate
algA
PMI
Mannose-6-phosphate
algC
PMM
Mannose-1-phosphate
algA
GMP
GDP-mannose
algD
GMD
GDP-mannuronic acid
ALGINATE

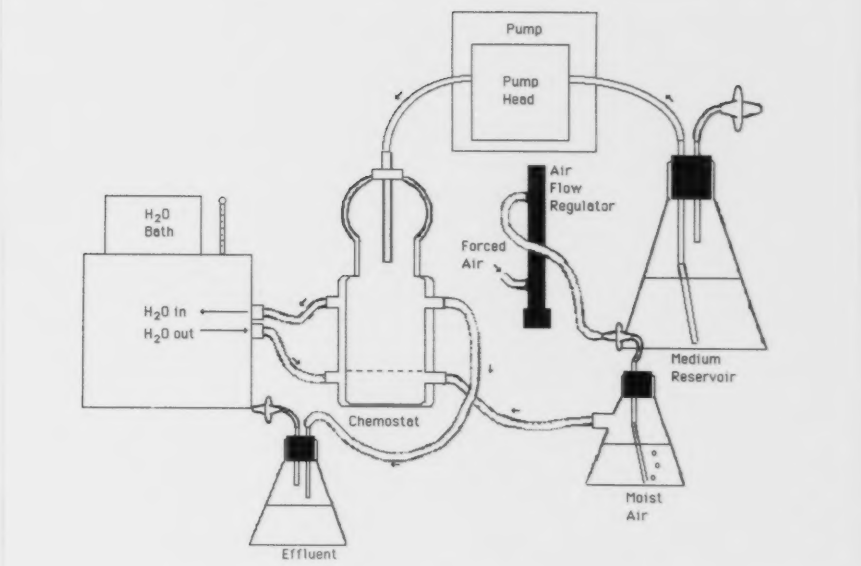
As already mentioned, high osmolarity (one of several potential environmental stress conditions) could be a signal leading to activation of the alginate biosynthetic genes in infecting *P. aeruginosa*, hence contributing to the emergence of the alginate-producing (mucoid) phenotype (Berry *et al.* 1989; Deretic *et al.*, 1987a; 1987b). Because mucoidy is a virulence factor associated with the colonization of the CF lung, the regulation of alginate expression has become a recent focus of investigation. The *algD* gene encodes GDP-mannose dehydrogenase (GMD) (Deretic *et al.*, 1987a; 1987b; 1987c; 1990; Deretic and Konyecsni, 1989a; 1989b), an NAD^+-dependent four electron transfer dehydrogenase which catalyzes the irreversible oxidation of GDP-mannose to GDP-mannuronic acid, an activated precursor for alginate synthesis (Roychoudry *et al.*, 1989; May *et al.*, 1991). The *algR* gene has been shown to positively control the expression of the *algD* gene (Deretic *et al.*, 1989; 1990; 1992). The transcriptional activation of *algD* has been shown to be necessary for alginate production, suggesting that the oxidation step catalyzed by GMD plays a major role in the commitment of cells to alginate production (Deretic *et al.*, 1987a; 1987b; DeVault *et al.*, 1989) and hence in the expression of the mucoid phenotype in *P. aeruginosa* (Deretic *et al.*, 1990; DeVault *et al.*, 1989).

As stated earlier, formation of a glycocalyx, of which alginate is an example, serves to protect organisms from both the host's immune system and from antibiotic therapy (Costerton *et al.*, 1987; May *et al.*, 1991). Elimination of the protective alginate layer of *P. aeruginosa* might render the bacterium more susceptible. This possibility prompted investigations into the effect of different environmental conditions on alginate production (Deretic *et al.*, 1987a; 1987b; 1987c; Davies *et al.*, 1993; Gacesa and Goldberg, 1992) as well

as the search for inhibitors of alginate synthesis (Costerton *et al.*, 1983; Elloumi *et al.*, 1992).

It is known that the ambient growth environment affects cells in many profound ways including their susceptibility to antibacterial agents (Brown and Williams, 1985; Costerton, 1988; Ellwood and Tempest, 1972). The interaction of sessile bacteria with antibiotics has been studied using *in vitro* chemostat systems which simulated growth conditions *in vivo* including slow growth rate and iron-restriction (Anwar *et al.*, 1989a; 1989b; Ombaka, 1983). The chemostat was designed to be an open system which could maintain cells at a specified growth rate under steady-state conditions. A schematic drawing of the system developed by Anwar *et al.* (1989a; 1989b) is shown in Plate 2. A brief description of the system is as follows: the desired temperature was maintained by recirculating water through the water jacket surrounding the chemostat vessel; nutrients were pumped into the chemostat vessel by a peristaltic pump set at the desired dilution rate, and because this was designed as a continuous culture system, culture was removed at the same rate as the addition of fresh nutrients; the effluent was collected in an effluent reservoir; and oxygen was supplied to the system in the form of humidified air bubbles. The *in vitro* chemostat provided an environment in which the interaction of planktonic and biofilm cells could be studied. Although the use of such a chemostat system simulates many *in vivo* conditions, there are limitations to its use and one is that oxygen is supplied in the form of air bubbles, which creates turbulence capable of removing loosely adhered bacterial cells. It is unlikely that bacterial biofilms *in vivo*, experience such turbulence.

PLATE 2. Schematic drawing of an *in vitro* chemostat system. This is an open system that can maintain cells growing at a specified growth rate under steady-state conditions. Temperature is maintained by recirculating water through the water jacket which surrounds the chemostat vessel. Nutrients are pumped into the vessel by means of a peristaltic pump set at a specific dilution rate. Culture is removed at the same rate as the addition of fresh nutrients. Oxygen is supplied to the system in the form of humidified air bubbles which also provides mixing. The ground glass plug is shown by the dashed line. Liquid level of the chemostat would be maximal at effluent port.


Pump
Pump Head
Air Flow Regulator
Forced Air
H_2O Bath
H_2O in
H_2O out
Medium Reservoir
Chemostat
Moist Air
Effluent

Therefore, it is possible that bacterial biofilms *in vivo* may be thicker than those observed using this system and therefore may be even more resistant to antibiotic therapy.

In a chemostat at least one essential nutrient is in a limiting concentration (that is, the organisms are nutrient-limited) (Bulder, 1992; Pirt, 1975). The rate of bacterial growth is regulated by controlling the rate of the addition of medium and the volumetric rate of nutrient addition is expressed as the dilution rate (Chakracklis, 1990; Pirt, 1975) which determines the time a bacterial cell remains in the chemostat (Pirt, 1975). Growth rate has been shown to affect the physiology, and in turn, the susceptibility of pathogenic microbes to antimicrobial agents *in vivo* (Brown and Williams, 1985; Cozens *et al.*, 1986; Eng *et al.*, 1991; Evans *et al.*, 1991; Gilbert *et al.*, 1990) and is therefore an important experimental parameter. The growth rate of biofilm cells is probably not strictly governed by dilution rate since these cells will probably have different access to limiting nutrients in comparison to planktonic cells. This might also be true for biofilm cells *in vivo*.

Through the use of the chemostat system described above, the influence of different environmental conditions on both planktonic and biofilm cells of *S. aureus* and *P. aeruginosa* was investigated. The ultimate aim of biofilm studies, such as those described in the following pages, is to develop the means by which the process of biofilm formation may be manipulated. Only when the mechanisms of biofilm formation are fully understood will the process be manageable.

II. MATERIALS AND METHODS

II.1 Materials

All culture media were from Difco.

All antibiotics were purchased from Sigma.

All other chemicals were of reagent grade.

Chemostats designed by Dr. H. Anwar were fabricated at Technical Services (Department of Chemistry, University of Alberta).

II.2 Bacterial strains and culture conditions

The bacterial strains used in the following investigations are listed in Table 1. All strains were maintained as freeze-dried cultures. Recovery was by overnight incubation at 37°C on blood agar plates for *Staphylococcus aureus* R 627; nutrient agar or *Pseudomonas* isolation agar (PIA) for the *Pseudomonas aeruginosa* strains. *P. aeruginosa* DMX 568/1 contains the *algD-xylE* transcriptional fusion on the chromosome. The transcriptional fusion was constructed by insertion of the *algD* (encodes for GDP-mannose dehydrogenase, GMP) promoter fragment upstream of the promoterless *xylE* gene (encodes catechol 2,3 dioxygenase, CDO) in a non-replicating vector and was inserted by homologous recombination into the chromosome of *P. aeruginosa* (V. Deretic, personal communication).

TABLE 1. Bacterial Strains

Organism	Strain	Characteristic	Reference
P. aeruginosa	ATCC 27853	non-mucoid	Anwar *et al.* (1991)
P. aeruginosa	492a	mucoid, CF isolate	Anwar *et al.* (1991)
P. aeruginosa	M 579	mucoid	Anwar *et al.* (1992)
P aeruginosa	DMX 568/1*	mucoid	
S. aureus	R 627	clinical isolate	Anwar *et al.* (1992)

* Received as a gift from Dr. V. Deretic (University of Texas Health Science Center at San Antonio, Texas)

II.3 Cultivation and sampling of bacteria in batch culture

For batch culture experiments, 100 mL volumes of broth were aseptically dispensed into duplicate sterile 500 mL Erlenmyer flasks covered with foam stoppers. After recovery of bacterial strains (from freeze-dry vials) on the appropriate solid medium in slants, 3 mL of fresh liquid medium was added to the slant to prepare a cell suspension. Flasks were inoculated with a 3% (v/v) inoculum. One millilitre aliquots were immediately removed for time zero samples and for purity checks. Inoculated flasks were placed on an orbital shaker set at the appropriate temperature and 250 rpm. One millilitre aliquots were removed at hourly intervals until the onset of stationary phase and then again at 24 hours for an additional 5 hours to obtain growth curves. Samples were treated as follows: optical density at 470 nm was measured using a Perkin Elmer Lambda 3 spectrophotometer; cells were pelleted in a microfuge for 5 minutes; supernatants were removed to fresh Eppendorf tubes; and both the cell pellets and the supernatants were kept at -20°C.

II.4 Cultivation of bacteria in the chemostat

II.4.1 Chemostat pump calibration

Double distilled and deionized water was used for the calibration. The delivery pipette from the top of the chemostat was placed into an empty beaker and water was siphoned out of the reservoir so that the tubing connecting the delivery pipette was completely filled with water (no air bubbles). The pump was allowed to run for 1 min to ensure there were no air

bubbles. At various pump settings, water was collected in a graduated cylinder for 5 min. At the end of the 5 min. intervals the volume of water collected was recorded as 'millilitres per 5 min.' and then converted to mL/min. A calibration curve was then prepared by plotting mL/min versus pump setting. In all cases the graph was linear. Care was always taken to calibrate the pump using the size of tubing that was to be used for the experimental chemostat set up.

II.4.2 Growth of chemostat cultures

The procedure for removal of iron from complex laboratory medium has been described previously (Kadurugamuwa *et al.*, 1987). Iron removal involved passing the medium through a column containing Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.). Of the iron present in complex laboratory medium, 85-95% was shown to be removed by this treatment. 2,2'-Dipyridyl (final concentration, 25 μg/mL) was added to the Chelex-treated medium to further restrict the availability of trace amounts of iron present in the medium. Since this method also removes other metal cations, 0.4 mM $MgSO_4$, 0.5 mM NaCl, and 0.62 mM KCl (final concentrations) were added to the medium to avoid imposing additional nutrient depletion on the cells.

A continuous culture system described by Anwar *et al.* (1989a, 1989b) was used. Chemostats with pieces of 1-cm (length, size 16) Masterflex silicone tubing (Cole-Parmer Instrument Ltd., Chicago, Ill.) were autoclaved at 121°C for 20 min. The lengths of silicone tubing serve as surfaces for the attachment of biofilms. Fresh medium was added to the chemostat using a peristaltic pump at a specific dilution rate as indicated in the Results. Five millilitres of bacterial culture grown to mid-exponential phase (OD_{470} = 1) in the

appropriate medium at 37°C was used as inoculum. Fresh medium was added to the culture at the dilution rate specified in the Results for each experiment.

II.4.3 Chemostat sampling and viable count determination

Chemostats were sampled from the top of the apparatus at the time intervals indicated in Results. Viable counts of planktonic bacteria in the suspension were determined using serially diluted samples incubated on nutrient agar (Difco, Detroit, Michigan) at 37°C. Two pieces of silicone tubing were removed for each biofilm sample. To quantitate biofilm cells adhered on the silicone tubing, the tubing was first washed three times with 10 mL phosphate-buffered saline (PBS, pH 7.4) to remove non-adherent bacterial cells, and then placed in 1 mL of PBS. The contents were vortex-mixed for 3 min. and the serially diluted samples were incubated on nutrient agar at 37°C. The efficiency of recovery of biofilm cells was determined by repeating the wash and plating steps. For the strains used, adherent cells were found to be about 99% recoverable. The colony forming units (CFUs) reported represent the lowest number possible (due to aggregation of cells).

II.5 Antibiotic administration

II.5.1 External to the chemostat system

Pieces of silicone tubing with adhered biofilm cells were removed from the chemostat, washed three times with PBS, and placed in 10 mL iron-depleted brain heart infusion (BHI-Fe) containing known concentrations of the antibiotics tobramycin, cephalexin, or both as indicated in the Results. For planktonic cells, 1 mL of the culture was diluted with BHI-Fe and transferred to BHI-Fe containing known concentrations of the antibiotics (10 mL total volume) to give 10^8 cells/mL. The same number of biofilm cells (as estimated from a curve of the kinetics of biofilm formation; Anwar *et al.* 1992b) were exposed to the known concentrations of the antibiotics. The samples were incubated at 37°C, and the viable counts of these samples were performed at timed intervals.

II.5.2 Within the chemostat vessel

Antibiotic stocks were prepared by dissolving antibiotic in 5 mL of medium. In all cases the antibiotics were prepared fresh daily and were sterilized by membrane filtration using a 0.22 μm filter. Antibiotics were added in doses at timed intervals corresponding to the dilution rate as indicated for each experiment. Samples were removed at intervals as specified in the Results section for each experiment and viable counts for both planktonic and biofilm cells were performed before the first dose of antibiotic was added to the chemostat and then just before the addition of each

subsequent dose of antibiotic. When antibiotic treatment was terminated fresh medium was continuously pumped into the chemostat at the same dilution rate for another three days to investigate regrowth of the organism.

II.6 Assays

II.6.1 Catechol 2,3 dioxygenase activity

The procedure of Nozaki (1970) was used as follows: cell pellets were resuspended in cold assay buffer containing 50 mM potassium phosphate buffer (pH 7.5) and 10% acetone (v/v); the suspension was sonicated 30 seconds on ice with the microtip probe of the Braun-Sonic 2000 (50 watts); the samples were then microfuged at 4°C and 13 000 rpm for 5 minutes; and then the resultant sonic cell-free extracts were assayed at room temperature in 1.0 mL reaction mixtures containing 50 mM phosphate buffer (pH 7.5), 0.3 mM catechol (Sigma) (the volume of the extract assayed varied from experiment to experiment but did not exceed 100 μL). The change in absorbance at 375 nm was followed using a Lambda 3 Spectrophotometer. One unit of catechol 2,3 dioxygenase (CDO) was defined as the amount of enzyme oxidizing 1 μmol of catechol to 2-hydroxymuconic semialdehyde (molar extinction coefficient of 4.4×10^4) per minute at room temperature.

II.6.2 Protein

Protein was assayed by the method of Bradford (1976) using Bio-Rad dye reagent according to the standard assay procedure. The protein standard used was bovine serum albumin.

II.6.3 Minimum inhibitory concentrations

Iron-depleted tryptic soy broth (TSB-Fe; Difco) was used in the determination of the minimum inhibitory concentration (MIC) of *P. aeruginosa* M 579 to the antibiotics tobramycin and piperacillin (Sigma Chemicals Co., St. Louis, Mo.). Cells grown overnight in liquid TSB-Fe were used as inoculum. The antibiotics were sterilized by membrane filtration. The MIC was determined by tube dilution (Kadurugamuwa *et al.* 1985). The lowest concentration of antibiotic resulting in the complete inhibition of visible growth was taken as the MIC.

II.7 Preparation of outer membranes and separation by polyacrylamide gel electrophoresis

Outer membranes were prepared by the method described by Filip *et al.* (1973). Briefly, the biofilm bacteria adherent to the silicone tubing were released by Vortex mixing for 3 minutes and the cells were harvested by centrifugation at 5000 x g for 10 min at 4°C. The bacterial pellet was suspended in 20 mL of distilled water and broken by 60-s pulses of sonication (Braun-Sonic 2000; 75 watts) ten times in an ice bath, with a 60-s interval for cooling. Unbroken cells were removed by centrifugation at 5000 x g for 10 min. Sarcosyl (N-lauryl sarcosinate, sodium salt; Sigma) was added to the

supernatant to a final concentration of 2%. The mixture was incubated for 30 minutes at room temperature and then centrifuged at 38 000 x g for 1 h. The membrane preparations were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (Blackshear, 1984) with a 14% acrylamide gel and ultrapure SDS (BDH Chemicals).

The molecular weights of proteins separated by SDS-PAGE were estimated by comparison with protein standards of known molecular weights. The protein standards used were phosphorylase *a* (97 400 Da), bovine albumin (66 000 Da), egg albumin (45 000 Da), pepsin (34 700 Da), trypsinogen (24 000 Da), and lysozyme (14 300 Da) (Sigma Chemicals).

III. RESULTS

III.1 The effect of tobramycin and cephalexin on *Staphylococcus aureus* R 627

Colonization of medical devices by biofilm forming bacteria represents a serious problem in modern therapeutic care. In order to compare the antibiotic susceptibility of planktonic and biofilm cells of *Staphylococcus aureus*, a common pathogen, an *in vitro* chemostat system was developed. For this investigation, two different approaches with respect to the administration of antibiotics were used. One involved antibiotic exposure external to the chemostat to address antibiotic susceptibility, and the other involved antibiotic administration within the chemostat to address not only susceptibility but also the influence of antibiotic clearing from the system. ß-Lactam (cephalexin) and aminoglycoside (tobramycin) antibiotics are often used in combination for the treatment of biofilm associated infections.

III.1.1 Antibiotic exposure external to the chemostat system

S. aureus R 627, isolated from an infected Tenckhoff catheter, was cultivated in a chemostat at a slow growth rate under iron limitation. Pieces of silicone tubing (1 cm lengths) were used as solid surfaces for biofilm formation. The dilution rate for these investigations was 0.05 h^{-1} which corresponds to one volume change of medium every 20 hours. To test for antibiotic susceptibility, both planktonic and biofilm samples were removed from the chemostat and transferred to antibiotic-containing iron-depleted brain-heart infusion (BHI-Fe) medium as described in the Materials and Methods section (II.8.1).

Prior to antibiotic susceptibility testing, adhesion and biofilm formation were addressed. Twenty-four hours after inoculation, the population of planktonic cells reached 5.5 x 10^9 cells/mL and remained relatively constant until termination of the experiment on day 13. Figure 2 illustrates the kinetics of biofilm formation by *S. aureus*. The number of biofilm CFUs increased exponentially from 6 x 10^4 to 2.7 x 10^7 cells/cm (length of silicone tubing) in the first 6 days and then continued to increase at a reduced rate. After day 11, the biofilm population reached a maximum density and remained relatively constant for the remainder of the experiment.

III.1.1.1 Susceptibility of planktonic cells to antibiotics

Antibiotic susceptibility of planktonic-phase cultures exposed to a constant concentration of antibiotics was achieved by exposing these cells to antibiotics external to the chemostat system. The sensitivity of planktonic cells of *S. aureus* harvested on day 13, to tobramycin (Fig. 3A), cephalexin (Fig. 3B), and a combination of these antibiotics (Fig. 3C) was determined. Greater than 100% survival in Figure 3A was observed due to growth of the organism in the medium in the absence of antibiotics (control). A rapid loss of cell viability occurred upon exposure of planktonic cells to tobramycin (2.5 and 5 μg/mL). The percentage of viable cells was reduced to less than 0.01% after a 5 h exposure to 2.5 μg/mL tobramycin (Fig. 3A). Increasing the concentration of tobramycin to 5 μg/mL resulted in an enhanced rate of killing of the planktonic cells. No viable cells were seen after 3 hours of incubation with the antibiotic.

FIGURE 2. Kinetics of biofilm formation by *Staphylococcus aureus* R 627 grown under iron-limitation in the chemostat ($D = 0.05\ h^{-1}$). (A similar figure appeared in Anwar *et al.*, 1992b).

(Figure 2 is adapted from Anwar et al. 1992B)

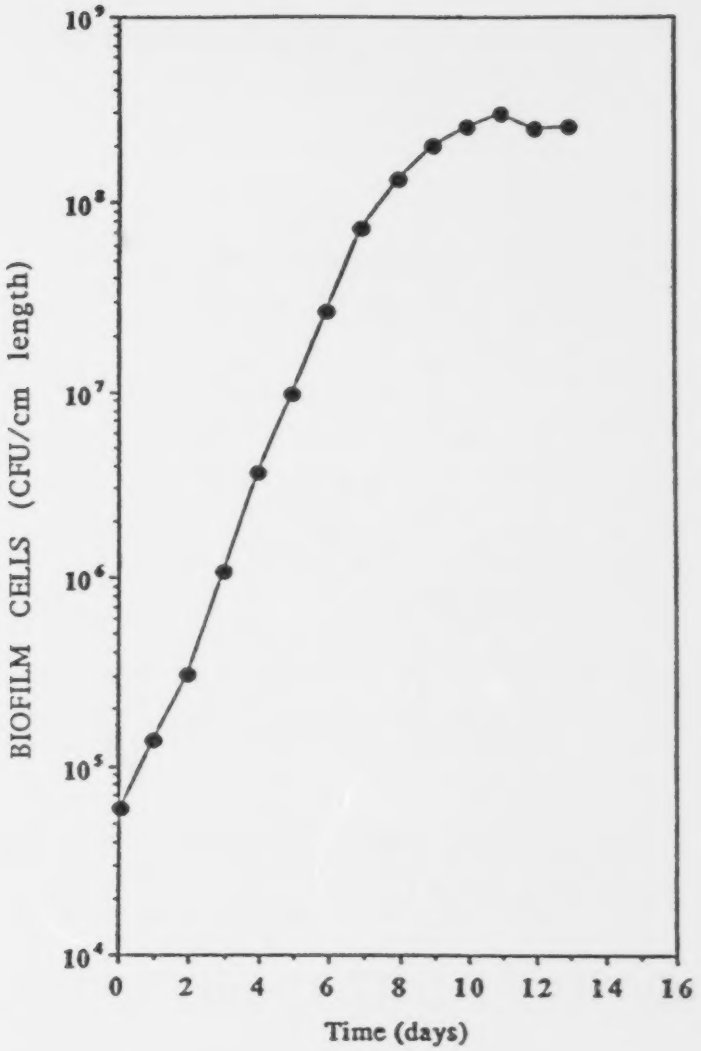
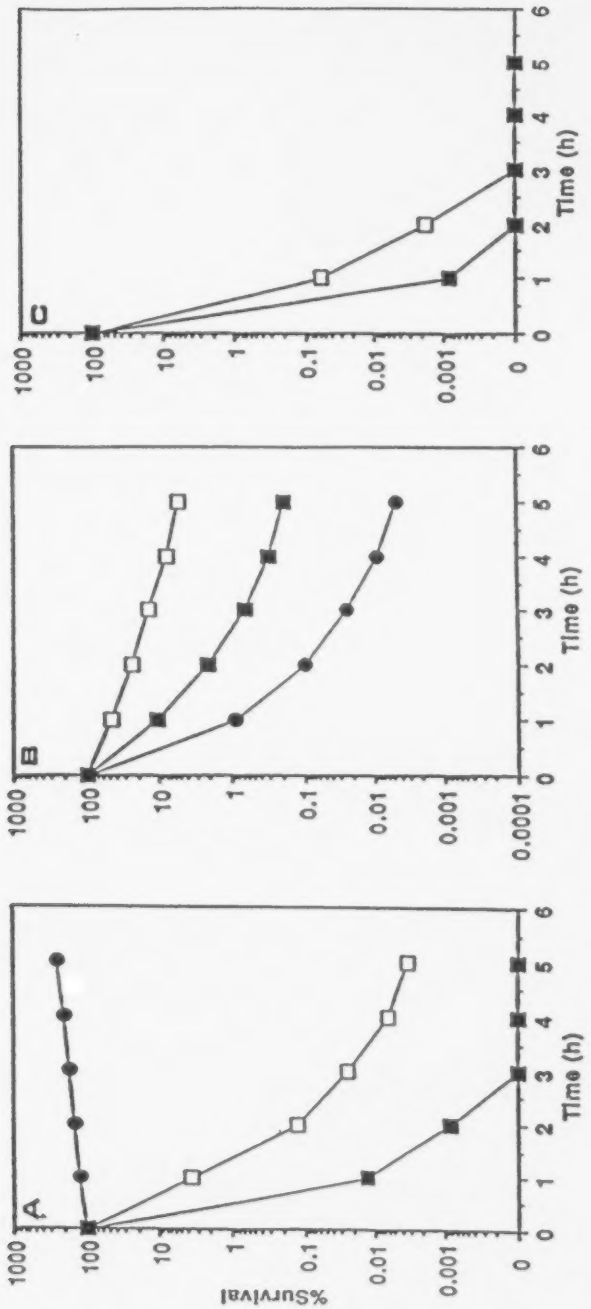

BIOFILM CELLS (CFU/cm length)
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
0
2
4
6
8
10
12
14
16
Time (days)

FIGURE 3. (A) Susceptibility of planktonic cells of *Staphylococcus aureus* R 627 grown in chemostat cultures (D = 0.05 h^{-1}) to tobramycin. Symbols: 0 μg tobramycin/mL (●); 2.5 μg tobramycin/mL (□); 5 μg tobramycin/mL (■). Note that greater than 100% survival is due to the growth of the organism in the absence of antibiotic (control). (B) Susceptibility of planktonic cells of *Staphylococcus aureus* R 627 grown in chemostat cultures (D = 0.05 h^{-1}) to cephalexin. Symbols: 25 μg cephalexin/mL (□); 50 μg cephalexin/mL (■); 100 μg cephalexin/mL (●). (C) Susceptibility of planktonic cells of *Staphylococcus aureus* R 627 grown in chemostat cultures (D = 0.05 h^{-1}) to a combination of tobramycin and cephalexin. Symbols: 2.5 μg tobramycin/mL plus 100 μg cephalexin/mL (□); 5 μg tobramycin/mL plus 100 μg of cephalexin/mL (■).

(A similar figure appeared in Anwar *et al.*, 1992b).

(Figure 3 is adapted from Anwar et al., 1992b.)


A
B
C
%Survival
Time (h)
Time (h)
Time (h)

The kinetics of killing of the planktonic cells of *S. aureus* by cephalexin is shown in Figure 3B. Cephalexin does not show the same level of killing activity against the planktonic cells as tobramycin. Higher concentrations of this compound have to be used to achieve killing. The viability was reduced from 100 to approximately 6% after a 5 h treatment with 25 μg/mL cephalexin. Increasing the concentration of cephalexin to 100 μg/mL resulted in a further reduction of planktonic cell viability. Approximately 0.005% of the planktonic cells were viable after a 5 h treatment of the cells with 100 μg of cephalexin per millilitre.

Figure 3C illustrates the susceptibility of planktonic S. *aureus* to combinations of tobramycin and cephalexin. Cephalexin was maintained at a concentration of 100 μg/mL (the highest concentration of cephalexin selected for this study). The viability of the planktonic cells was rapidly reduced when the cells were treated with 2.5 μg tobramycin plus 100 μg cephalexin per millilitre. No viable cells were recovered after a 3 h exposure to these concentrations of antibiotics. Increasing the concentration of tobramycin from 2.5 μg/mL to 5 μg/mL in combination with 100 μg/mL cephalexin, resulted in an enhanced rate of killing of the planktonic cells. When tobramycin was administered in combination with cephalexin, activity against planktonic *S. aureus* was improved as compared with tobramycin alone.

III.1.1.2 Susceptibility of young and old biofilm cells to antibiotics

Biofilm cells of *S. aureus* were defined as 'young' when they were harvested on day 4 and as 'old' when they were harvested on day 13. An equivalent number of young and old biofilm cells were exposed to known concentrations of tobramycin and cephalexin.

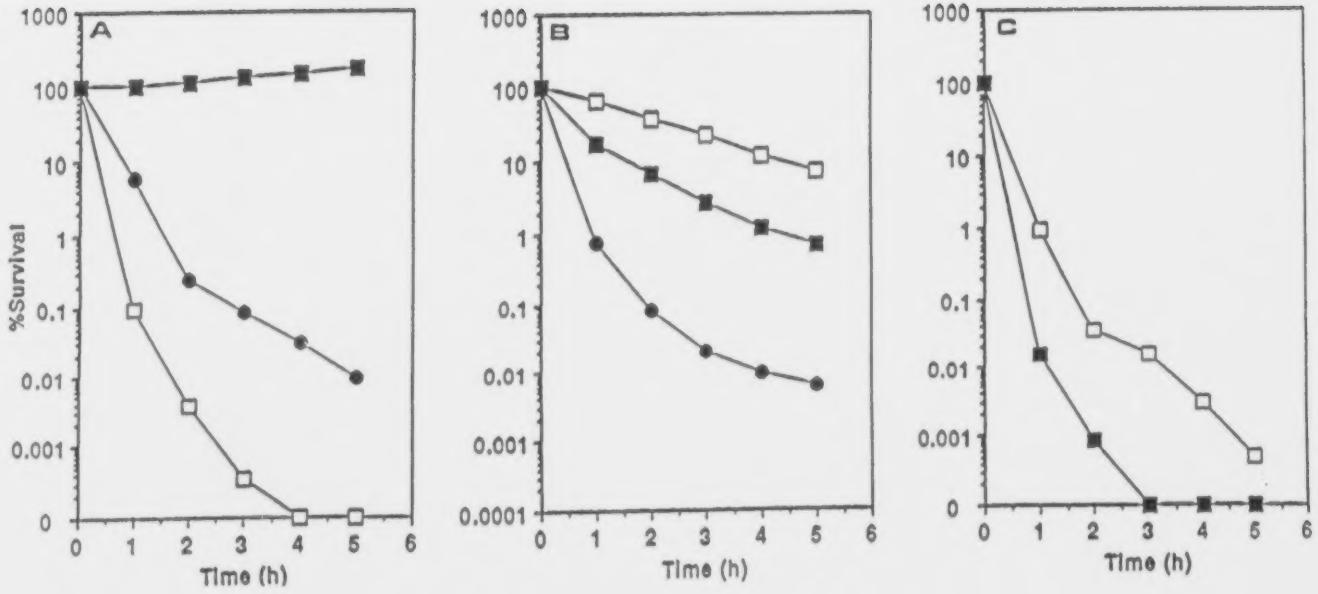
The interaction of young biofilm cells of *S. aureus* with tobramycin is illustrated in Figure 4A. Young biofilm cells were sensitive to tobramycin. Greater than 100% survival in Figure 4A was due to growth of the organism in the absence of antibiotics. A reduction in cell viability to less than 0.1% was observed after exposure to 2.5 μg tobramycin per millilitre for 5 h. Increasing the concentration of tobramycin to 5 μg/mL resulted in a more rapid reduction of cell viability (no viable cells were recovered after 4 h) .

Figure 4B illustrates the effect of cephalexin on young biofilm cells of *S. aureus*. Exposure to 25 μg of cephalexin per millilitre results in a steady decline in the viability of the young biofilm cells. Increasing the cephalexin concentration to 50 μg/mL resulted in enhanced activity. A 5 h exposure of young biofilm cells to 25 μg cephalexin per millilitre reduced cell viability to 6%. When the concentration of cephalexin was increased to 50 μg/mL, the percentage of survival decreased to less than 1%. When cephalexin concentration was increased to 100 μg/mL, viability was reduced to less than 0.01%. Cephalexin does not appear to have as high a level of activity against young biofilm cells of *S. aureus* as does tobramycin.

The susceptibility of young biofilm cells of *S. aureus* to the combination of tobramycin and cephalexin is shown in Figure 4C. A rapid

FIGURE 4. (A) Kinetics of killing of young biofilm cells of *Staphylococcus aureus* R 627 grown in the chemostat (D = 0.05 h^{-1}) by tobramycin. Symbols: 0 μg tobramycin/mL (■); 2.5 μg tobramycin/mL (●); 5 μg tobramycin/mL (□). Note that greater than 100% survival is due to the growth of the organism in the absence of antibiotic (control). (B) Kinetics of killing of young biofilm cells of *Staphylococcus aureus* R 627 grown in the chemostat (D = 0.05 h^{-1}) by cephalexin. Symbols: 25 μg cephalexin/mL (□); 50 μg cephalexin/mL (■); 100μg cephalexin/mL (●). (C) Kinetics of killing of young biofilm cells of *Staphylococcus aureus* R 627 grown in the chemostat (D = 0.05 h^{-1}) by a combination of tobramycin and cephalexin. Symbols: 2.5 μg tobramycin/mL plus 100 μg cephalexin/mL (□); 5 μg tobramycin/mL plus 100 μg of cephalexin/mL (■).
(A similar figure appeared in Anwar *et al.*, 1992b).

(Figure 4 is adapted from Anwar et al., 1992b).

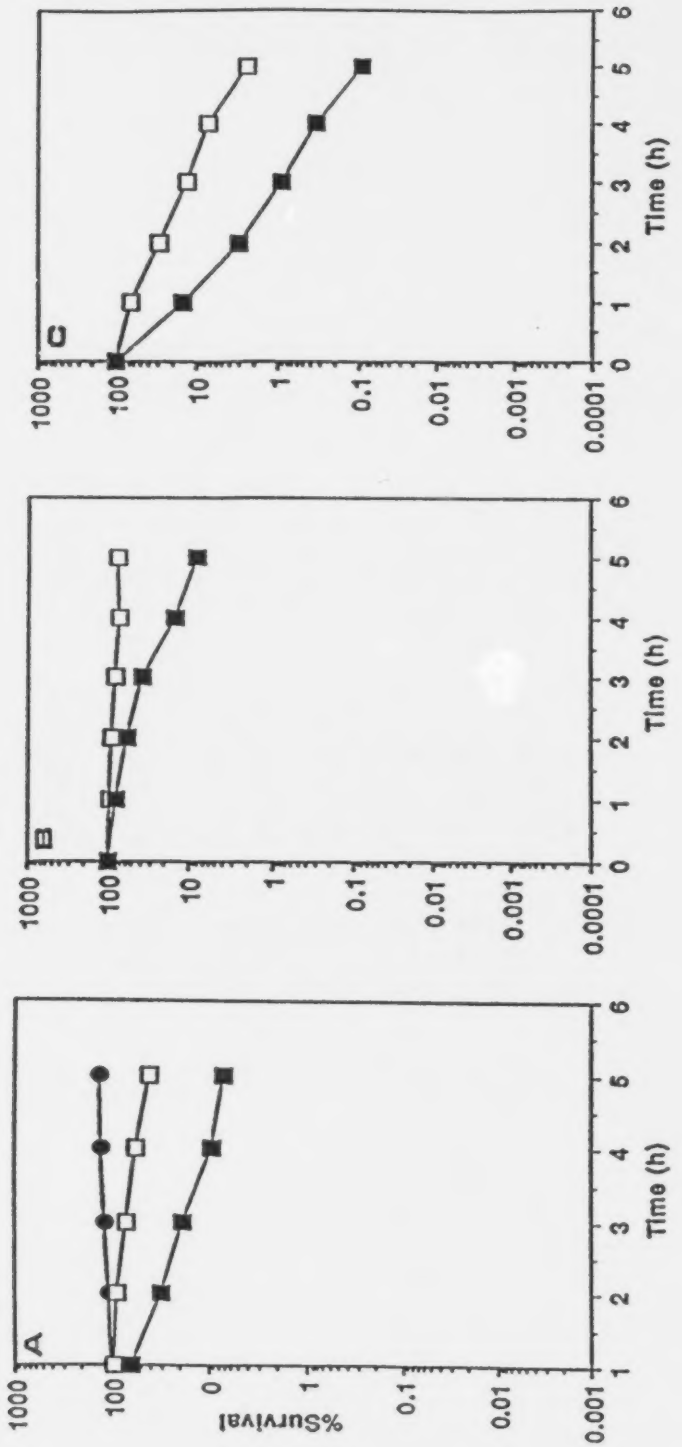

A
B
C
%Survival
Time (h)
Time (h)
Time (h)
1000
100
10
1
0.1
0.01
0.001
0.0001
0
0 1 2 3 4 5 6

loss of cell viability resulted from treatment of the young biofilm cells with tobramycin plus cephalexin. An enhanced rate of killing of young biofilm cells was observed when tobramycin was combined with cephalexin. After treatment with 2.5 μg tobramycin plus 100 μg cephalexin per millilitre for 5 h, the cell viability dropped to 0.0005%. Increasing the concentration of tobramycin to 5 μg/mL while maintaining the concentration of cephalexin at 100 μg/mL enhances the bactericidal effect, and no viable cells were recovered after a three hour exposure.

Tobramycin has less effect on old biofilm cells of *S. aureus* than on young biofilm (Fig. 5A). Greater than 100% survival in Figure 5A reflects growth of the organism in the control medium which is devoid of antibiotics. A tobramycin concentration of 2.5 μg/mL was unable to reduce cell viability. Survival of old biofilm cells of *S. aureus* experiencing a 5 h exposure to 5 μg tobramycin per millilitre decreased to approximately 40% (Fig. 5A), whereas survival of young biofilm cells undergoing the same treatment was reduced to less than 0.0001% (Fig. 4A). When the concentration of tobramycin was further increased to 10 μg/mL, an enhanced rate of killing of the old biofilm cells of *S. aureus* was observed. Viability of old biofilm cells was reduced to 7% when treated for 5 h with 10 μg tobramycin per millilitre. Old biofilm cells released from silicone tubing by mixing on a Vortex mixer before exposure to tobramycin showed the same susceptibilities observed with planktonic cells (Fig. 3A).

The interaction of old *S. aureus* biofilm cells with cephalexin is illustrated in Figure 5B. Old biofilm cells were found to be significantly more resistant than young biofilm cells (Fig. 4B) to the bactericidal effects of cephalexin. A cephalexin concentration of 50 μg/mL was ineffective

FIGURE 5. (A) Interaction of old biofilm cells of *Staphylococcus aureus* R 627 grown in chemostat cultures (D = 0.05 h^{-1}) with tobramycin. Symbols: control (no antibiotic) (●); 5 μg tobramycin/mL (□); 10 μg tobramycin/mL (■). Note that greater than 100% survival is due to the growth of the organism in the absence of antibiotic (control). (B) Interaction of old biofilm cells of *Staphylococcus aureus* R 627 grown in the chemostat (D = 0.05 h^{-1}) by cephalexin. Symbols: 50 μg cephalexin/mL (□); 100 μg cephalexin/mL (■). (C) Interaction of old biofilm cells of *Staphylococcus aureus* R 627 grown in the chemostat (D = 0.05 h^{-1}) by a combination of tobramycin and cephalexin. Symbols: 5 μg tobramycin/mL plus 100 μg cephalexin/mL (□); 10 μg tobramycin/mL plus 100 μg of cephalexin/mL (■).
(A similar figure appeared in Anwar *et al.*, 1992b).
Figure 5 is adapted from Anwar et al., 1992b).


A
B
C
%Survival
Time (h)
1000
100
10
1
0.1
0.01
0.001
0.0001
0
1
2
3
4
5
6

against the old biofilm cells. Increasing the concentration of cephalexin to 100 μg/mL resulted in an enhanced rate of killing activity although a significant proportion of the cells remained viable. A 5 h exposure to this concentration reduces viability to about 20%. Old biofilm cells were susceptible to cephalexin when they were released from the silicone tubing by Vortex mixing.

As is shown in Figures 4 and 5, old biofilm is quite resistant to killing when either tobramycin and cephalexin is used alone. The susceptibility of old *S. aureus* biofilm cells to the combination of tobramycin and cephalexin is shown in Figure 5C. A steady decline in cell viability resulted from a 5 h exposure of the old biofilm cells of *S. aureus* to 5 μg tobramycin plus 100 μg cephalexin per millilitre, and survival dropped to approximately 2.5%. Increasing the concentration of tobramycin to 10 μg/mL while maintaining the concentration of cephalexin at 100 μg/mL enhances killing of the old biofilm cells and survival was reduced to approximately 0.09%. Therefore the combination of tobramycin with cephalexin resulted in an enhanced level of killing of old biofilm cells of *S. aureus*.

III.1.2 Antibiotic administration within the chemostat system

For these studies, *S. aureus* R 627 was cultivated in a chemostat as described above (III.1.1). However, in contrast to the studies outlined in III.1.1 where the biofilms were removed from the chemostat and exposed to fixed antibiotic concentrations, in this case, antibiotics were added directly into the chemostat vessel. Antibiotic addition in this way allows one to observe how clearing of antibiotics from the culture vessel affects the organism over time. The dilution rate for these investigations was 0.17 h^{-1} which corresponds to

one volume change of BHI-Fe medium every 6 hours. This corresponds to the frequency of administration of clinical doses of these antibiotics to patients receiving peritoneal catheters. As with the previous experiment, the cells were cultivated under iron-restriction. For the purposes of this study, young biofilms were defined as those grown for 2 days, while old biofilms were defined as those grown for 21 days. After the initial dose (loading dose) was administered to the chemostat, samples were removed at hourly intervals for 6 h, and viable counts were obtained for both planktonic and biofilm cells as outlined in Material and Methods (II.4.3). At 6 h intervals thereafter, maintenance (subsequent) doses were added. Viable counts were determined before the addition of each maintenance dose as described in Material and Methods (II.4.3 and II.6.2).

III.1.2.1 Effect of chemostat-controlled loading doses of antibiotic on planktonic and biofilm cells

The effect of the loading-dose combination of tobramycin (70 μg/mL) and cephalexin (500 μg/mL) on young and old biofilm cells of *S. aureus* is illustrated in Figures 6A and 6B, respectively. The loading-dose corresponds to the first dose of antibiotic administered into the chemostat. Planktonic and young biofilm cells were very susceptible to these drug levels (Fig. 6A). The survival of planktonic cells was reduced to approximately 0.0002% following a 6 h exposure to these antibiotics (Fig. 6A). The population of young biofilm cells was reduced from 100% to approximately 0.005% following a similar exposure (Fig. 6A).

FIGURE 6. Kinetic interaction of planktonic and biofilm cells of the 2-day-old (A) and 21-day-old (B) chemostat cultures of *Staphylococcus aureus* R 627 with tobramycin and cephalexin at levels representing the loading dose (70 and 500 μg/mL respectively). The dilution rate (0.17 h^{-1}) was chosen so that one volume change of the medium in the chemostat occurred in 6 h. Symbols: planktonic cells (○); biofilm cells (●).

(A similar figure appeared in Anwar *et al.*, 1992d).

(Figure 6 is adapted from Anwar et al., 1992d).

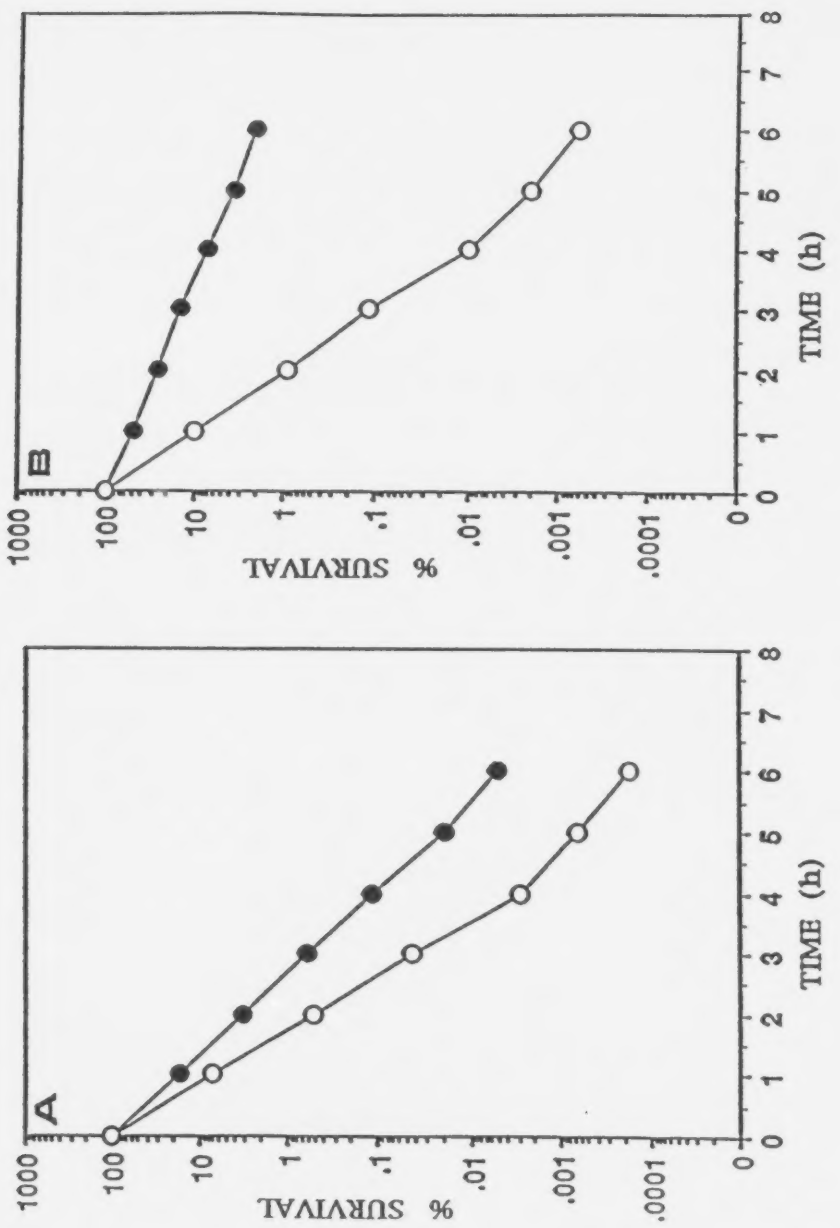
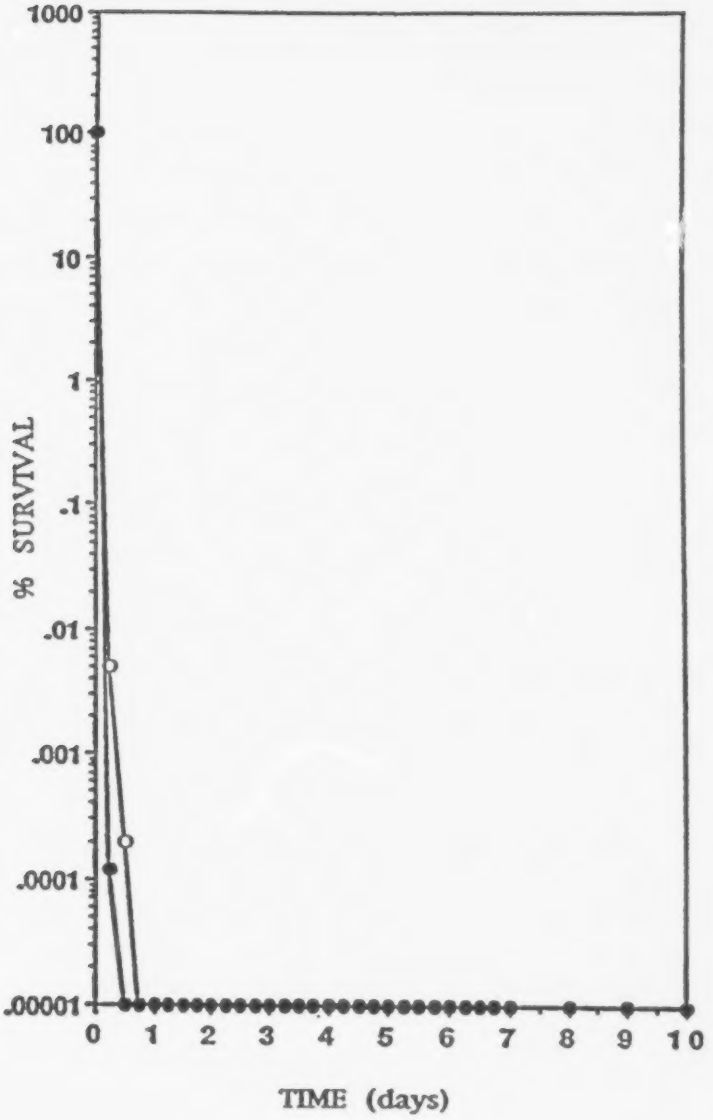

A
B
% SURVIVAL
TIME (h)
1000
100
10
1
.1
.01
.001
.0001
0
1
2
3
4
5
6
7
8

Figure 6B shows the interaction of planktonic and old biofilm cells with the loading doses of tobramycin and cephalexin. Planktonic cells of the 21-day-old chemostat culture were still sensitive to these doses of tobramycin and cephalexin. As with the 2-day-old chemostat culture discussed above, the survival of the planktonic population was reduced to approximately 0.0005% following exposure to these antibiotic doses. However, in contrast, the 21 day-old biofilm cells were much more resistant than young biofilm cells and their associated planktonic cells (Fig. 6B). The viability of old biofilm cells was reduced to about 2.5% following exposure to the loading doses of these antibiotics for 6 hours.

III.1.2.2 Interaction of planktonic and young biofilm cells with tobramycin and cephalexin

Figure 7 illustrates the interaction of planktonic and young biofilm (2 days old) cells with drug levels representing the loading and maintenance doses. The initial dose (70 μg tobramycin and 500 μg cephalexin per mL) was called the loading dose and the subsequent doses (62 μg tobramycin and 250 μg cephalexin per mL) were called the maintenance doses. The loading dose was administered 2 days after inoculation of the chemostat. Maintenance doses were administered at 6-hour intervals for 7 days. The survival of the planktonic population dropped to approximately 0.0001% within 6 hours after exposure to the loading dose. There were no viable young biofilm cells recovered after exposure to the second maintenance dose. Antibiotic administration was continued for one week as it might be done in a clinical setting. Neither planktonic nor young biofilm cells could be recovered from

FIGURE 7. Kinetic interaction of planktonic and young biofilm cells of the 2-day-old chemostat culture of *Staphylococcus aureus* R 627 with tobramycin and cephalexin at levels representing the loading (70 μg of tobramycin and 500 μg cephalexin per mL) and maintenance (62.5 μg of tobramycin and 250 μg cephalexin per mL) doses of these antibiotics. The initial (first) dose was the loading dose, and those following were maintenance doses. The dilution rate (0.17 h^{-1}) corresponds to one volume change of the medium in the chemostat every 6 hours. Symbols: planktonic cells (●); biofilm cells (○).
(A similar figure appeared in Anwar *et al.*, 1992d).
(Figure 7 has been adapted from Anwar et al., 1992d).


1000
100
10
1
.1
.01
.001
.0001
.00001
% SURVIVAL
0 1 2 3 4 5 6 7 8 9 10
TIME (days)

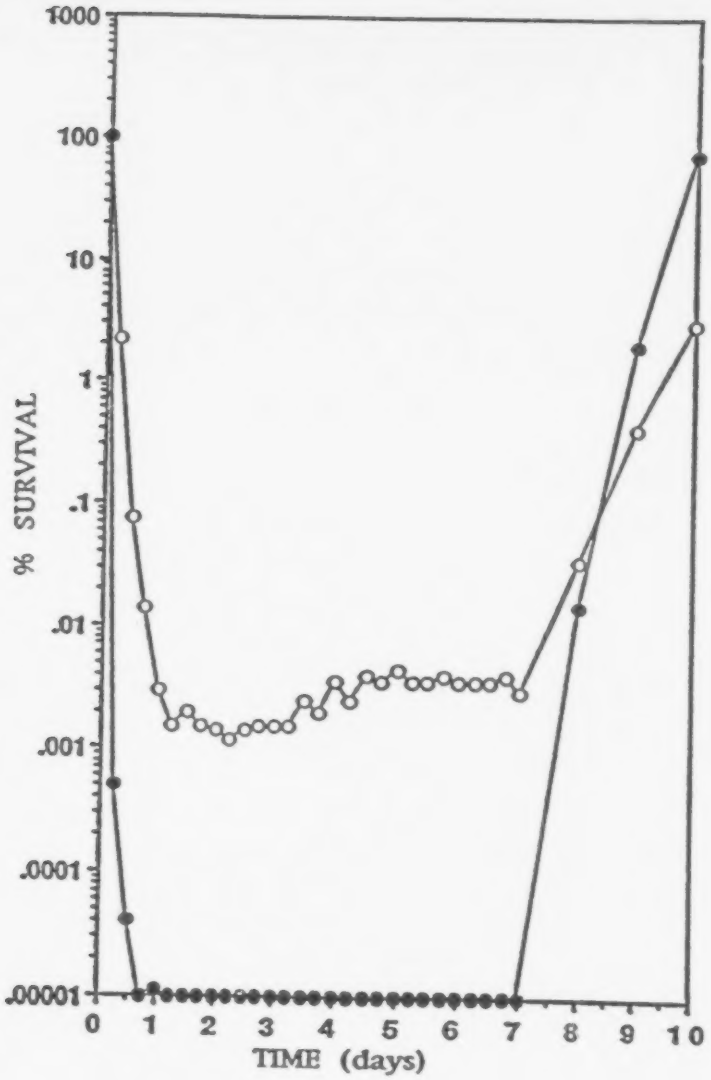
the chemostat during this period. Samples were removed for 3 days after antibiotic treatment was discontinued on day 7; no viable planktonic nor young biofilm cells were recovered.

III.1.2.3 Interaction of planktonic and old biofilm cells with antibiotics

Figure 8 shows the kinetic interaction of 21 day-old planktonic and old biofilm cells with loading and maintenance doses of tobramycin and cephalexin. The loading dose was administered on day 21 and maintenance doses were administered at 6-h intervals. The old biofilm population was reduced to 2.5%, while that of planktonic cells dipped to 0.005% after experiencing the same antibiotic exposure (loading dose). The survival of old biofilm cells decreased to 0.08% after exposure to the first maintenance dose, and further decreased to approximately 0.002% after exposure to four maintenance doses. The percent viability remained constant at 0.002% (approximately 1000 viable CFU/cm length) even when maintenance doses were administered for an additional six days. On day 7, the antibiotic treatment was discontinued and fresh antibiotic-free BHI-Fe medium was pumped into the chemostat for 3 an additional three days. The planktonic population returned to 100% within three days after antibiotic treatment was withdrawn. Viability of old biofilm cells increased to 2% during the same time period. These results show that the loading and maintenance doses of tobramycin and cephalexin used in this study failed to eliminate old *S. aureus* biofilms.

FIGURE 8. Kinetic interaction of planktonic and old biofilm cells of the 21-day-old chemostat culture of *Staphylococcus aureus* R 627 with tobramycin and cephalexin at levels representing the loading (70 μg of tobramycin and 500 μg of cephalexin per mL) and maintenance (62.5 μg of tobramycin and 250 μg of cephalexin per mL) doses of these antibiotics. The initial (first) dose was the loading dose, and those following were maintenance doses. The dilution rate (0.17 h^{-1}) corresponds to one volume change of the medium in the chemostat every 6 hours. Symbols: planktonic cells (●); biofilm cells (○).
(A similar figure appeared in Anwar *et al.*, 1992d).

(Figure 8 has been adapted from Anwar et al., 1992d).


1000
100
10
1
.1
.01
.001
.0001
.00001
% SURVIVAL
0 1 2 3 4 5 6 7 8 9 10
TIME (days)

III.2 Growth characteristics and expression of iron-regulated outer-membrane proteins of chemostat-grown *Pseudomonas aeruginosa*

At infection sites, pathogenic bacteria are likely to be growing under nutrient limitation at sub-optimal growth rates and must be able to adapt if they are to survive. In the chemostat, dilution rate is equivalent to the growth rate under steady-state conditions. The use of a chemostat therefore enables one to study the influence of both growth rate and nutrient limitation on biofilm formation. In the experiment described below, the chemostat system was used to study the growth and the expression of iron-regulated outer-membrane proteins of biofilm cells of *Pseudomonas aeruginosa* ATCC 27853 (non-mucoid strain) and *Pseudomonas aeruginosa* 492a (mucoid strain) cultivated under conditions of iron limitation. A mucoid and non-mucoid strain were used in order to determine the extent to which mucoidy influences biofilm formation.

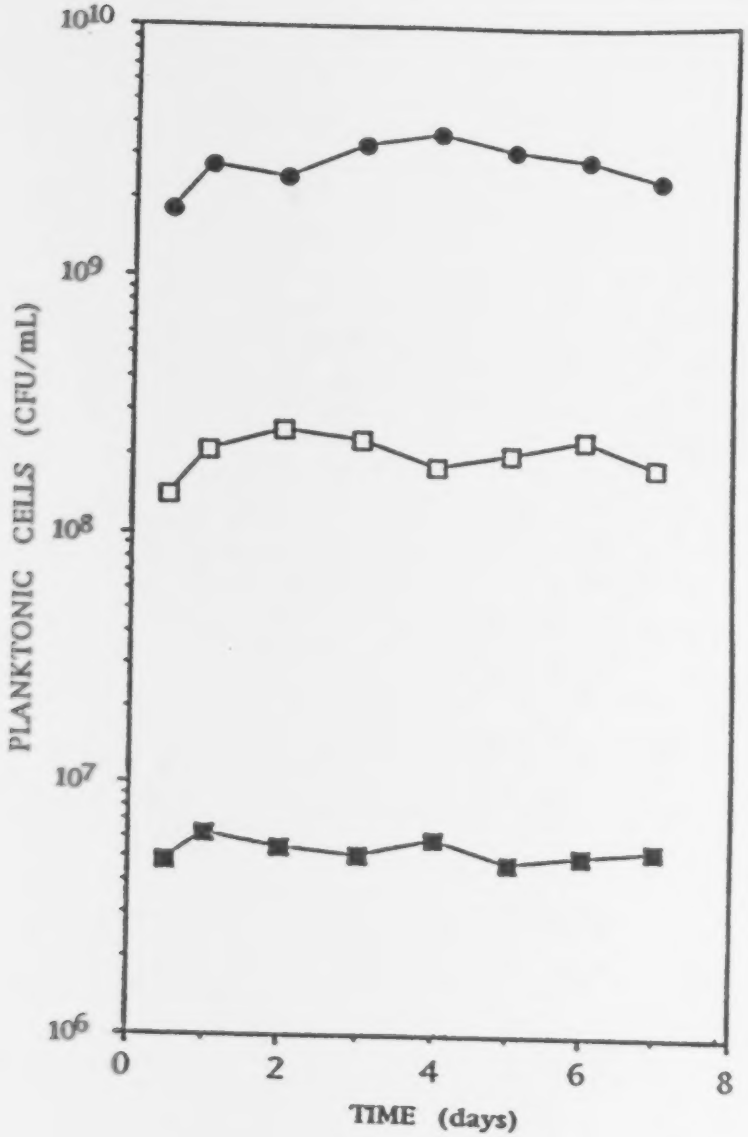
III.2.1 Kinetics of growth and biofilm formation of mucoid and non-mucoid *Pseudomonas aeruginosa*

P. aeruginosa was cultivated in a chemostat at different dilution rates as described in Material and Methods (II.4.2). Figure 9 shows the growth kinetics of mucoid *P. aeruginosa* planktonic cells in the chemostat. Dilution rate (D) influenced the number of planktonic cells present in the chemostat; the population remained relatively constant for any given dilution rate studied. Both mucoid and non-mucoid planktonic populations remained at about 3 x 10^9 cells/mL when the dilution rates were increased from 0.05 hr^{-1} to 0.2 hr^{-1}. In this range of dilution rates, the cells were not washed out. A decrease

FIGURE 9. The effect of dilution rate (D) on the planktonic population of a mucoid strain of *Pseudomonas aeruginosa* (492a). Symbols: D = 0.05 or 0.2 h^{-1} (●); D = 0.5 h^{-1} (□); D = 1.0 h^{-1} (■). The y-axis represents colony-forming units (CFU) per millilitre.

(A similar figure appeared in Anwar *et al.*, 1991).

(Figure 9 has been adapted from Anwar et al., 1991).


10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
PLANKTONIC CELLS (CFU/mL)
0
2
4
6
8
TIME (days)

in the population of the planktonic cells was observed when the dilution rate was increased to 0.5 hr^{-1}. The number of planktonic cells dropped to 2 x 10^8 cells/mL and a new steady state was established. Increasing the dilution rate to 1.0 hr^{-1} decreased cell numbers of both mucoid and non-mucoid *P. aeruginosa* to 5 x 10^6 cells/mL. At high dilution rates, some of the planktonic cells observed may have been due to the release of loosely adhered biofilm cells from the lengths of silicone tubing (see Figs. 10A and 10B).

Biofilm formation by both non-mucoid (Figure 10A) and mucoid (Figure 10B) strains of *P. aeruginosa* increased as the dilution rate was increased. At a dilution rate of 0.05 hr^{-1}, there was an exponential increase of non-mucoid *P. aeruginosa* colonizing the silicone tubing between day 1 and day 5 (Fig. 10A) reaching 9 x 10^7 CFU/cm (length) and remaining fairly constant until the experiments were terminated on day 7 (Fig 10A). When the dilution rate was increased to 0.5 hr^{-1} (Fig. 10A), the biofilm population reached 5 x 10^8 CFU/cm (length) on day 3 and remained constant until day 7. Although a rapid decline in the number of planktonic cells was observed when the dilution rate was increased to 1.0 hr^{-1} (Fig. 9), an increase in the number of biofilm cells colonizing the silicone tubing was observed (Fig. 10A). The number of biofilm cells of the non-mucoid strain increased exponentially at this dilution rate, from day 1 to day 3 reaching 1.4 x 10^9 CFU/cm (length). From the third day onward, the sessile population slowly increased to 2 x 10^9 CFU/cm (length) by day 7. The growth rate of biofilm cells is probably not strictly governed by the dilution rate, since these cells may have different access to limiting nutrients in comparison with planktonic cells. Mucoid *P. aeruginosa* seemed better able to form biofilms (based on the number of

FIGURE 10. Kinetics of biofilm formation by (A) the non-mucoid *Pseudomonas aeruginosa* (ATCC 27853) and (B) the mucoid *P. aeruginosa* 492a at different dilution rates (D). Symbols: D = 0.05 h^{-1} (●); D = 0.2 h^{-1} (○); D = 0.5 h^{-1} (■); D = 1.0 h^{-1} (□). The y-axis represents colony-forming units (CFU) per centimetre length of the silicone tubing.

(A similar figure appeared in Anwar *et al.*, 1991).

(Figure 10 has been adapted from Anwar et al. 1991).

A
B
BIOFILM CELLS (CFU/cm length)
TIME (days)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
0
2
4
6
8

colony forming units) than the non-mucoid strains (compare Fig. 10A and 10B).

The effect of excess iron on planktonic cell growth and biofilm formation was investigated in separate experiments. This was done to ensure that no expression of IROMPs occurred in the presence of iron. An excess supply of iron was supplied to cells in the chemostat by the addition of 10 μM $FeSO_4$ to the medium reservoir. The growth kinetics of the planktonic cells were similar to those observed in Figure 9. The number of biofilm cells colonizing the silicone tubing increased when medium containing 10 μM $FeSO_4$ was pumped into the chemostat at a dilution rate of 0.2 hr^{-1} (Figs. 11A and 11B). At a dilution rate of 0.2 hr^{-1} (with 10 μM $FeSO_4$) the profile of biofilm formation (Figs. 11A and 11B) was similar to the profile observed for a dilution rate of 0.5 hr^{-1} (no $FeSO_4$) in Figures 10A or 10B.

III.2.2 Relationship between dilution rate and the populations of planktonic and biofilm cells

The effects of dilution rate on planktonic and biofilm populations in the chemostat is shown in Figure 12. Viable count data taken from day 7 was used in the construction of this graph. The populations of planktonic cells remained relatively constant at 2-3 x 10^9 cells/mL when the dilution rates were changed from 0.05 hr^{-1} to 0.2 hr^{-1}. The number of planktonic

FIGURE 11. Kinetics of biofilm formation by (A) the non-mucoid *Pseudomonas aeruginosa* (ATCC 27853) and (B) the mucoid *P. aeruginosa* 492a at different dilution rates (D). Symbols: D = 0.2 h^{-1} (●); D = 0.5 h^{-1} (■); D = 1.0 h^{-1} (□). The y-axis represents colony-forming units (CFU) per centimetre length of the silicone tubing. $FeSO_4$ (10 μM) was added to the medium reservoir.

(A similar figure appeared in Anwar *et al.*, 1991).

(Figure 11 has been adapted from Anwar et al., 1991).

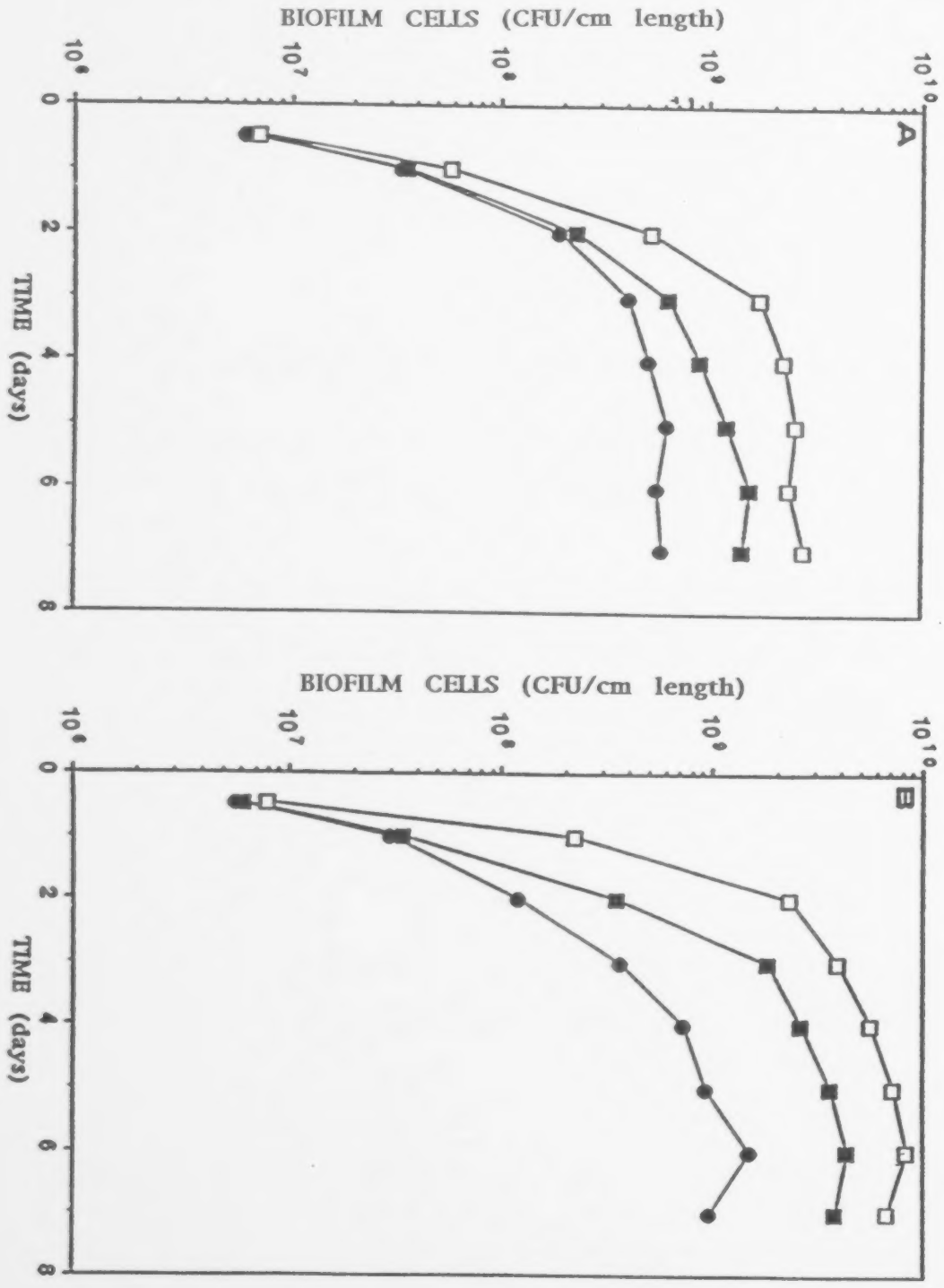
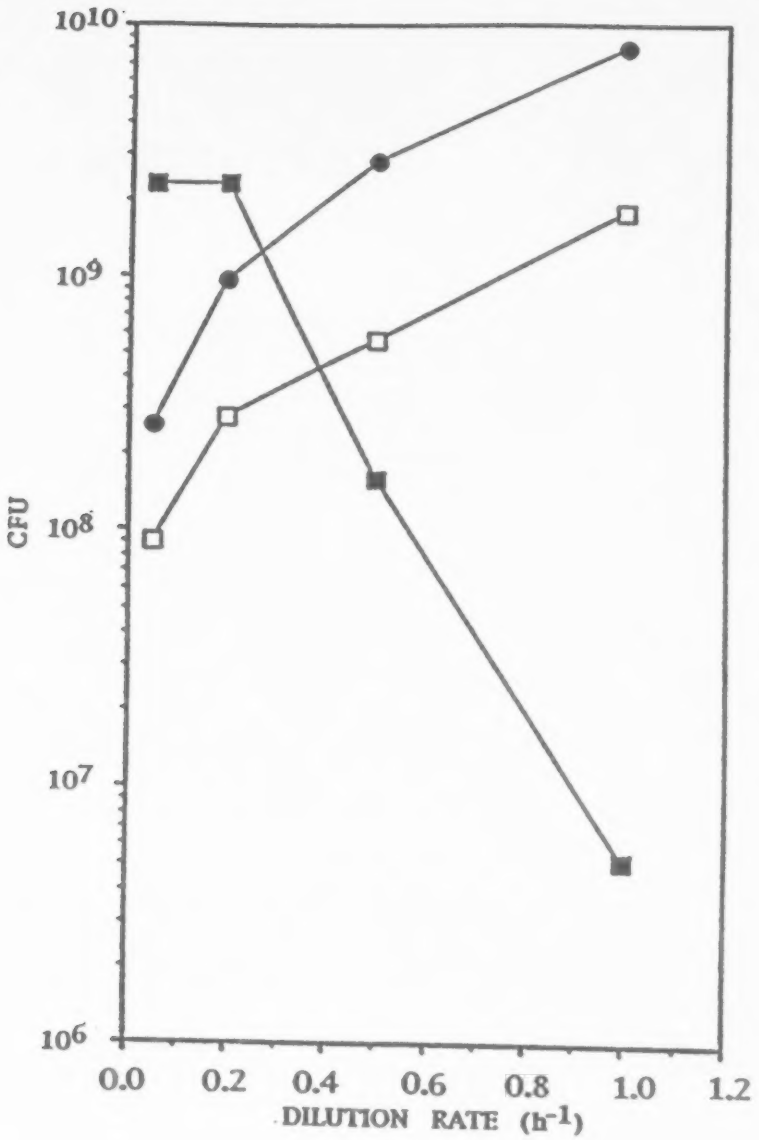

BIOFILM CELLS (CFU/cm length)
10⁶
10⁷
10⁸
10⁹
10¹⁰
0
2
4
6
8
TIME (days)
A
BIOFILM CELLS (CFU/cm length)
10⁶
10⁷
10⁸
10⁹
10¹⁰
0
2
4
6
8
TIME (days)
B

FIGURE 12. Relationship between the dilution rate and the populations of planktonic and biofilm cells of *Pseudomonas aeruginosa*. Symbols: Planktonic cells of mucoid *P. aeruginosa* 492a or non-mucoid *P. aeruginosa* ATCC 27853 (■); biofilm cells of mucoid *P. aeruginosa* 492a (●); biofilm cells of nonmucoid *P. aeruginosa* ATCC 27853 (□).

(A similar figure appeared in Anwar *et al.*, 1991).

(Figure 12 was adapted from Anwar et al., 1991).


10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
CFU
0.0
0.2
0.4
0.6
0.8
1.0
1.2
DILUTION RATE (h^{-1})

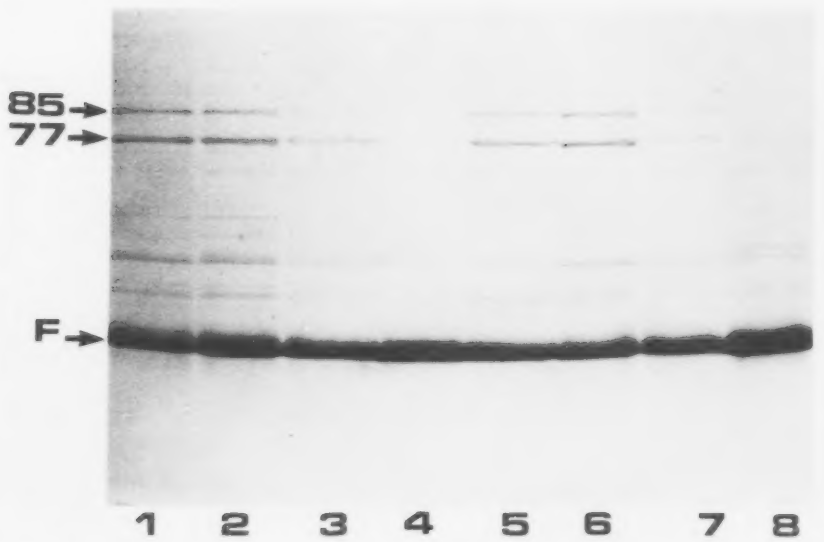
cells dropped to 2 x 10^8 cells/mL when the dilution rate was increased to 0.5 hr^{-1}. Increasing the dilution rate to 1.0 hr^{-1} caused a dramatic decrease in the number of planktonic cells to 5 x 10^6 cells/mL.

The number of the biofilm cells of both mucoid and non-mucoid *P. aeruginosa* increased as the dilution rate was increased. This is opposite of what was observed for the populations of planktonic cells in the chemostat. Viable counts of biofilm cells of mucoid *P. aeruginosa* were consistently higher than those of the non-mucoid strain. The number of mucoid biofilm cells colonizing the silicone tubing on day 7 was 2.5 x 10^8 CFU/cm (length) at the dilution rate of 0.05 hr^{-1} and this figure increased to 8 x 10^9 CFU/cm (length) when the dilution rate was increased to 1.0 hr^{-1}. The number of non-mucoid biofilm cells was 9 x 10^7 CFU/cm (length) when the dilution rate was 0.05 hr^{-1} and approximately 2 x 10^9 CFU/cm (length) when the dilution rate was increased to 1.0 hr^{-1}.

III.2.3 Expression of iron-regulated outer-membrane proteins of biofilm cells of *Pseudomonas aeruginosa*

During iron limitation, cells normally induce the expression of outer membrane proteins involved in the transport of iron-siderophore complexes, to sequester iron required for metabolic activities. In order to show this for biofilm cells of *P. aeruginosa*, the effect of dilution rate on the expression of iron-regulated outer-membrane proteins (IROMPs) in biofilm cells of both mucoid and non-mucoid strains is shown in Plate 3. Similar outer membrane protein profiles were observed for planktonic cells (data not shown). Expression of IROMPs was affected by the dilution rate. As expected, the IROMPs (77K and 85K) were induced when the dilution rates were low (0.05

PLATE 3. SDS-PAGE of IROMPs of biofilm cells of mucoid and non-mucoid strains of *Pseudomonas aeruginosa* (ATCC 27853 and 492a) cultivated at different dilution rates (D) in the chemostat. Lanes 1-4, *P. aeruginosa* ATCC 27853. Lane 1, D = 0.05 h^{-1}; lane 2, D = 0.2 h^{-1}; lane 3, D = 0.5 h^{-1}; lane 4, D = 1.0 h^{-1}. Lanes 5-8, *P. aeruginosa* 492a. Lane 5, D = 0.05 h^{-1}; lane 6, D = 0.2 h^{-1}; lane 7, D = 0.5 h^{-1}; lane 8, D = 1.0 h^{-1}. Numbers at left refer to molecular weights (x 10^3). A total of 40 μg of protein was loaded into each lane. F is the porin of *P. aeruginosa* as described by Hancock *et al.* (1990). (A similar figure appeared in Anwar *et al.*, 1991).


85
77
F
1 2 3 4 5 6 7 8

and 0.20 hr^{-1}). At a higher dilution rate of 0.5 hr^{-1} the expression of IROMPs was still detectable but became barely detectable when the dilution rate was increased to 1.0 hr^{-1}. It has been previously shown that the 77K and 85K OM proteins of *P. aeruginosa* were the iron-regulated outer membrane proteins expressed when the organism was cultivated under iron restriction (Anwar *et al.* 1984; Brown *et al.* 1984; Ward *et al* . 1988). The outer membrane of biofilm cells cultivated in the presence of 10 μM $FeSO_4$ at a dilution rate of 0.20 hr^{-1} was also analyzed and found to be identical to those observed in Plate 3, lanes 4 and 8. These cells did not express the 77K and 85K outer membrane proteins.

III.3 Interaction of mucoid *Pseudomonas aeruginosa* with tobramycin and piperacillin

Pseudomonas aeruginosa is an important opportunistic pathogen. In its mucoid form, this organism persists and appears to be less susceptible to antibiotic treatment. This problem is compounded by the biofilm mode of growth. The susceptibility of planktonic, young, and old biofilm cells of mucoid *P. aeruginosa* M 579 to ß-lactam (piperacillin) and aminoglycoside (tobramycin) antibiotics was compared. Cultivation of this organism was as described above (II.4.2). The dilution rate of the chemostat was set to 0.125 h^{-1} so that one volume change of the medium in the chemostat occurred every 8 hours. This corresponds to the frequency of clinical administration of these antibiotics.

III.3.1 Kinetics of biofilm formation by *Pseudomonas aeruginosa* M 579

The planktonic population of mucoid *P. aeruginosa* reached 4 x 10^9 cells per mL and remained constant until tobramycin and piperacillin were administered into the chemostat. Figure 13 illustrates the kinetics of biofilm formation of this strain of *P. aeruginosa*. The adherent bacterial population increased exponentially from day 1 to day 5 reaching 1.5 x 10^9 CFU per cm (length) and continued to increase at a slower rate to reach 2.5 x 10^9 CFU per cm (length) on day 10. Turbulence created by the supply of oxygen in the form of air bubbles was expected to release some of the loosely adhered bacterial cells from the lengths of silicone tubing. This might contribute to the equilibrium state established from day 7 to day 10 (Fig. 13).

III.3.2 Reaction of planktonic and young biofilm cells to the first chemostat-controlled dose of antibiotics

For the purposes of this study, 'young' biofilm cells were 2-day old sessile cultures while 'old' biofilm cells were sessile cells cultivated for 10 days in the chemostat.

The susceptibility of young biofilm cells of mucoid *P. aeruginosa* to 500 μg of piperacillin per mL plus 5 μg of tobramycin per mL is illustrated in Figure 14. Cell viability was monitored every 2 h for 8 hours after the initial dose of antibiotic was administered. Tobramycin concentration was kept at 5 μg/mL because this is believed to be achievable in serum. Both planktonic and young biofilm cells of this mucoid strain were sensitive to the concentrations of tobramycin and piperacillin used and the percentages of survival of planktonic and young biofilm cells were 0.01 and 0.45%, respectively (Fig. 14).

FIGURE 13. Kinetics of biofilm formation of *Pseudomonas aeruginosa* M 579 grown slowly under iron-restricted conditions in the chemostat. The dilution rate (0.125 h^{-1}) corresponds to one volume change of the medium every 8 h.

(A similar figure appeared in Anwar *et al.*, 1992a).

(Figure 13 has been adapted from Anwar et al., 1992a).

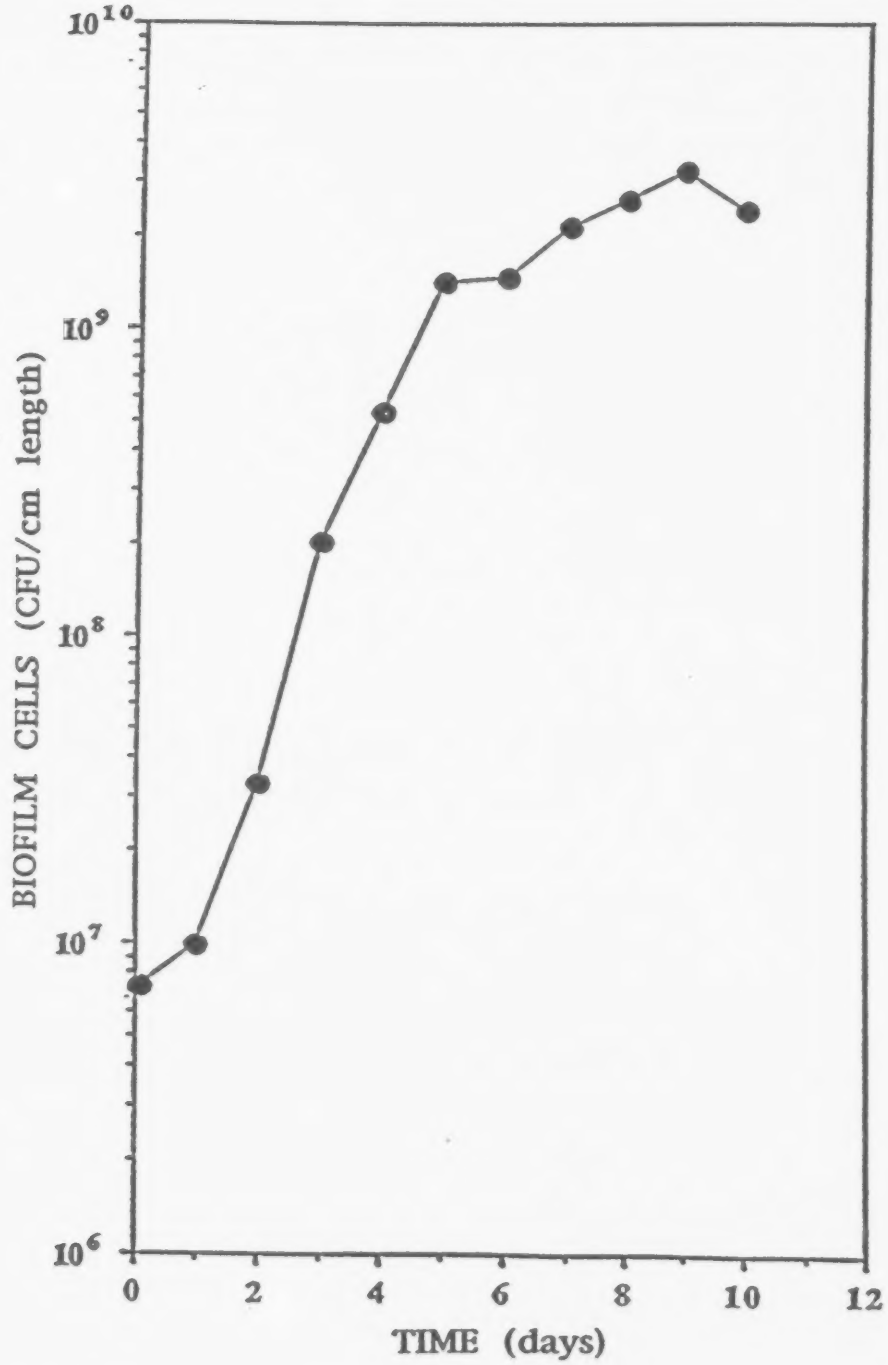
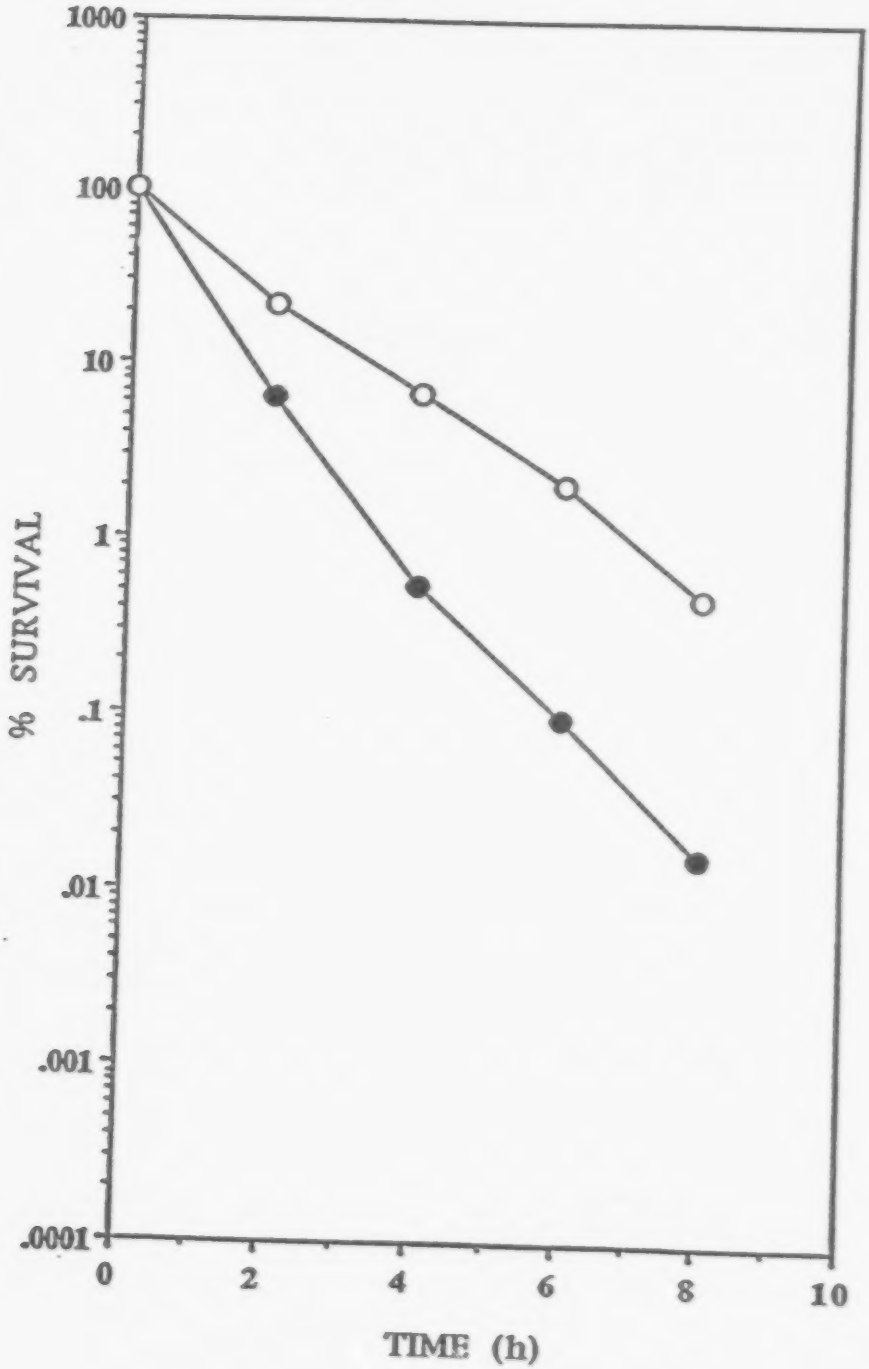

10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
BIOFILM CELLS (CFU/cm length)
0
2
4
6
8
10
12
TIME (days)

FIGURE 14. Interaction of planktonic and young biofilm cells of the 2-day-old chemostat cultures of mucoid *Pseudomonas aeruginosa* M 579 with the first dose of 500 μg of piperacillin plus 5 μg of tobramycin per mL. The dilution rate (0.125 h^{-1}) corresponds to one volume change of the medium every 8 h. Symbols: planktonic cells (●); biofilm cells (○).
(A similar figure appeared in Anwar *et al.*, 1992a).
(Adapted from Anwar et al)


1000
100
10
1
.1
.01
.001
.0001
% SURVIVAL
0
2
4
6
8
10
TIME (h)

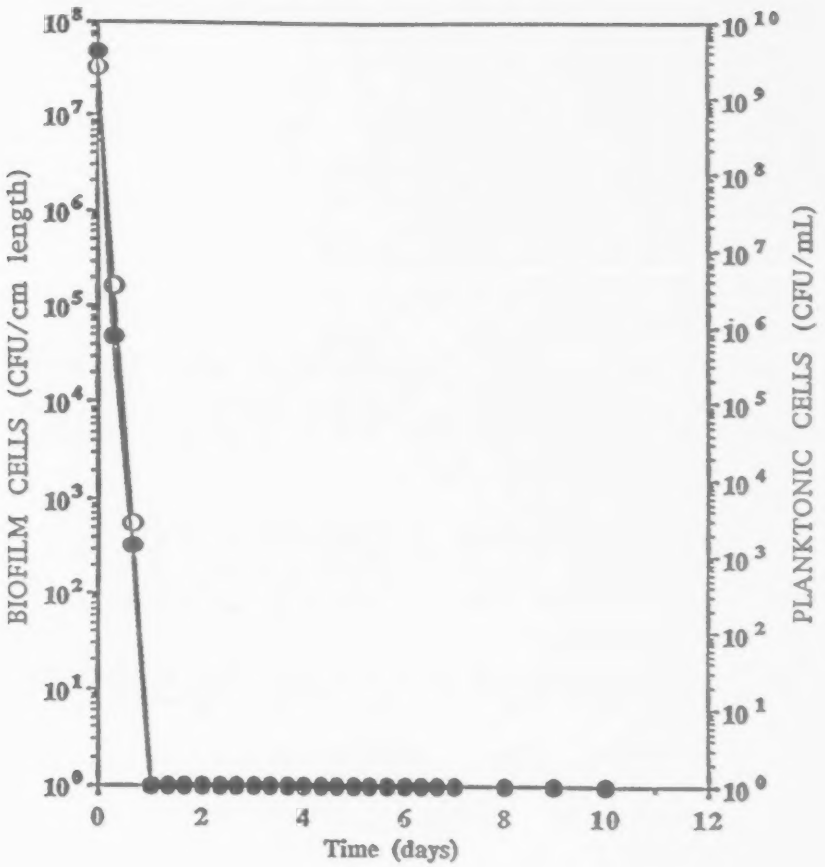
III.3.3 Interaction of planktonic and young biofilm cells with tobramycin and piperacillin

Preliminary studies showed that both planktonic and young biofilm cells of *P. aeruginosa* were sensitive to chemostat-controlled doses of 5 μg of tobramycin per mL plus 250 μg of piperacillin per mL. Planktonic and young biofilm cells were completely killed after exposure to six maintenance doses of tobramycin plus piperacillin. When antibiotic therapy was continued for an extra 5 days, no viable planktonic or young biofilm cells were recovered during this period. Regrowth of the organism did not occur after antibiotic treatment was discontinued on day 7. Figure 15 shows the effect of a seven day dosing regimen of tobramycin plus piperacillin on planktonic and young biofilm cells of *P. aeruginosa*. Only a marginal increase in the activity of these antibiotics resulted from increasing the concentration of piperacillin to 500 μg/mL (Fig. 15).

III.3.4 Interaction of planktonic and old biofilm cells with tobramycin and piperacillin

Ten day-old chemostat cultures of *P. aeruginosa* M 579 showed heavy colonization of the lengths of silicone tubing; even the silicone tubing connecting the effluent port of the chemostat to the waste reservoir showed heavy colonization. These old biofilm cells were exposed to 250 μg of piperacillin per mL plus 5 μg of tobramycin per mL or 500 μg of piperacillin per mL plus 5 μg of tobramycin per mL. Viability was monitored every 2 h for the first 8 h and then at 8 h intervals as before. The viability of the planktonic and old biofilm cells was not significantly reduced after exposure to the first dose of these antibiotics. After the first dose of tobramycin and piperacillin,

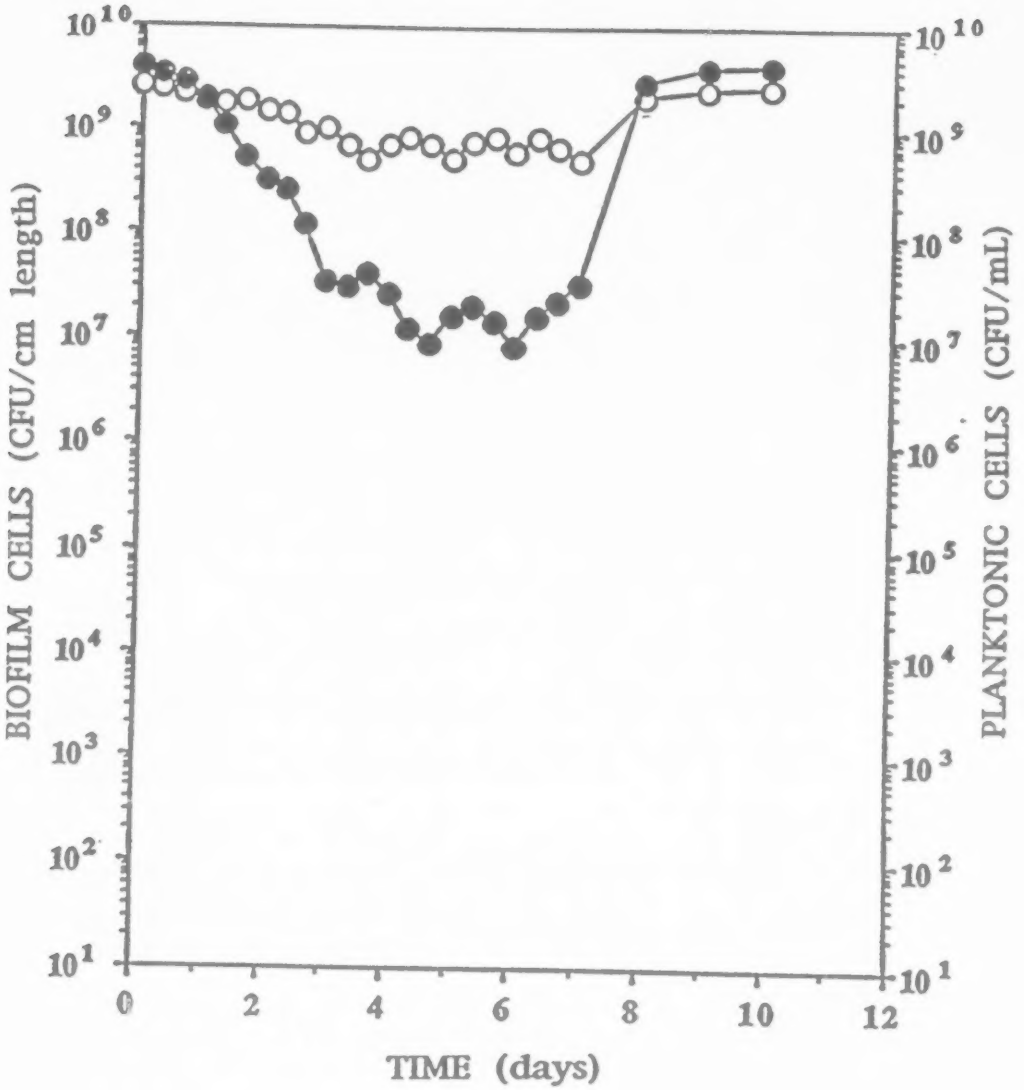
FIGURE 15. Interaction of planktonic and young biofilm cells of the 2-day-old chemostat cultures of mucoid *Pseudomonas aeruginosa* M 579 with doses of 500 μg of piperacillin per mL plus 5 μg of tobramycin per mL at 8-hour intervals for 7 days. Symbols: planktonic cells (●); biofilm cells (○).
(A similar figure appeared in Anwar *et al.*, 1992a).
(Adapted from Anwar et al)


BIOFILM CELLS (CFU/cm length)
PLANKTONIC CELLS (CFU/mL)
Time (days)
0
2
4
6
8
10
12

two different colonial types were detected on nutrient agar: one was opaque and the other was transparent. Each colony type was streaked onto *Pseudomonas* isolation agar (PIA) to ensure that the chemostat culture was not contaminated, and both the opaque and the transparent colonies grew. Opaque colonies, resembling the original culture, comprised about 80% of the colonies observed on a given plate, while the remaining 20% were transparent. To further confirm that these two colony types represented the original culture, outer membrane protein profiles were prepared and compared. These profiles were identical for the two colony types. Opaque colonies were more resistant to both tobramycin and piperacillin than transparent colonies. The minimum inhibitory concentrations (MICs) of tobramycin for opaque and transparent colonies were 0.6 μg/mL and 0.15 μg/mL, respectively. The MICs of piperacillin for opaque and transparent colonies were 26 and 6.5 μg/mL, respectively. These different colony types were observed in relatively the same proportion even after discontinuation of antibiotic treatment. Different colony types were not observed with the young biofilm cells nor with the planktonic cells associated with them.

Figure 16 illustrates the effect of multiple doses of 500 μg of piperacillin per mL plus 5 μg of tobramycin per mL on old biofilm of *P. aeruginosa* for a period of seven days. Preliminary studies had indicated that old biofilm cells and their associated planktonic cells were very resistant to the combination of 250 μg of piperacillin per mL plus 5 μg of tobramycin per mL. The interaction of planktonic and old biofilm cells of mucoid *P. aeruginosa* with the combination of 500 μg of piperacillin plus 5 μg of tobramycin per mL was tested (Figure 16). These concentrations of piperacillin and tobramycin were not adequate to completely kill old biofilm cells. The planktonic

FIGURE 16. Interaction of planktonic and old biofilm cells of the 10-day-old chemostat cultures of mucoid *Pseudomonas aeruginosa* M 579 with doses of 500 μg of piperacillin per mL plus 5 μg of tobramycin per mL at 8-hour intervals for 7 days. Symbols: planktonic cells (●); biofilm cells (○).
(A similar figure appeared in Anwar *et al.*, 1992a).
(Adapted from Anwar et al)


BIOFILM CELLS (CFU/cm length)
PLANKTONIC CELLS (CFU/mL)
TIME (days)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
0
2
4
6
8
10
12

population increased to 100% after antibiotic treatment was discontinued. This is probably due to the release of some of the old biofilm population into the planktonic phase.

III.4 Effect of growth environment on *algD* transcription in *Pseudomonas aeruginosa* DMX 568/1

The structural gene *algD* encodes GDP-mannose dehydrogenase (GMP), a key enzyme in the alginate biosynthetic pathway. *P. aeruginosa* DMX 568/1 contains a chromosomally located transcriptional fusion of the *algD* promoter to the promoterless *xylE* reporter gene which codes for catechol 2,3 dioxygenase (CDO). CDO catalyzes the formation of muconic semialdehyde from catechol and was used to monitor *algD* transcription. Since alginate production contributes significantly to the virulence of *P. aeruginosa*, investigation of environmental conditions influencing the expression of *algD*, may lead to a better understanding of how to control *P. aeruginosa* infections. To begin to address this, a series of experiments using solid medium and batch culture were performed.

III.4.1 Effect of salt concentration on *algD* transcription in *Pseudomonas aeruginosa* grown on solid medium

The activation of *algD* as measured by catechol 2,3 dioxygenase reporter gene activity, has been previously shown to be responsive to high osmolarity when grown on agar plates (Deretic *et al.*, 1990). Therefore the high osmolarity in the CF lung is one of several potential environmental conditions that may be activating *algD* transcription. In order to confirm this observation, *algD* expression in *P. aeruginosa* DMX 568/1 was tested. Since

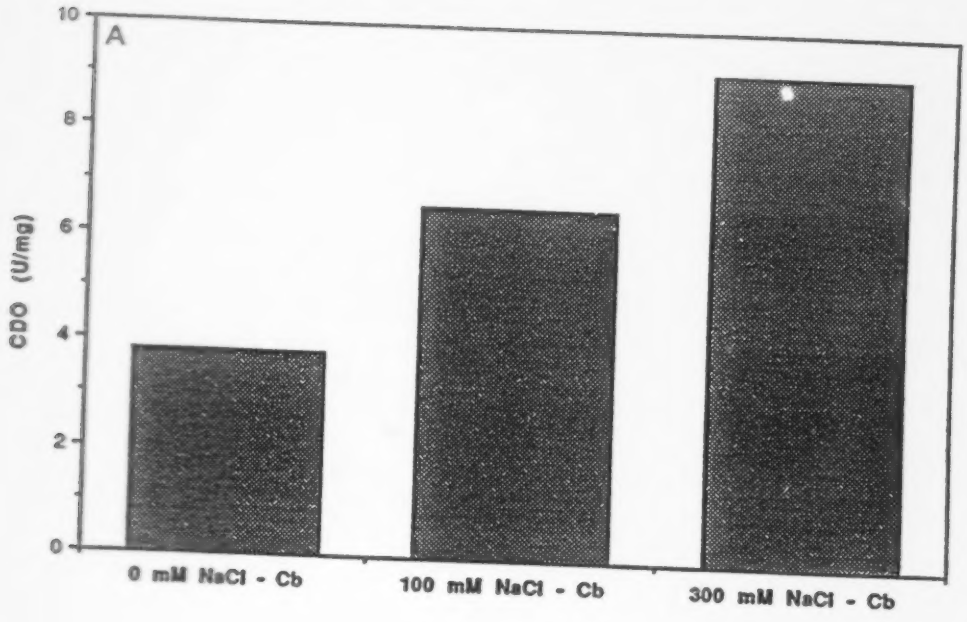
carbenicillin was thought to be required for the maintenance of the *algD/xylE* transcriptional fusion (the vector used for construction of the chromosomal fusion contained the carbenicillin resistance gene as a marker), the effect of carbenicillin addition on *algD* transcription was investigated.

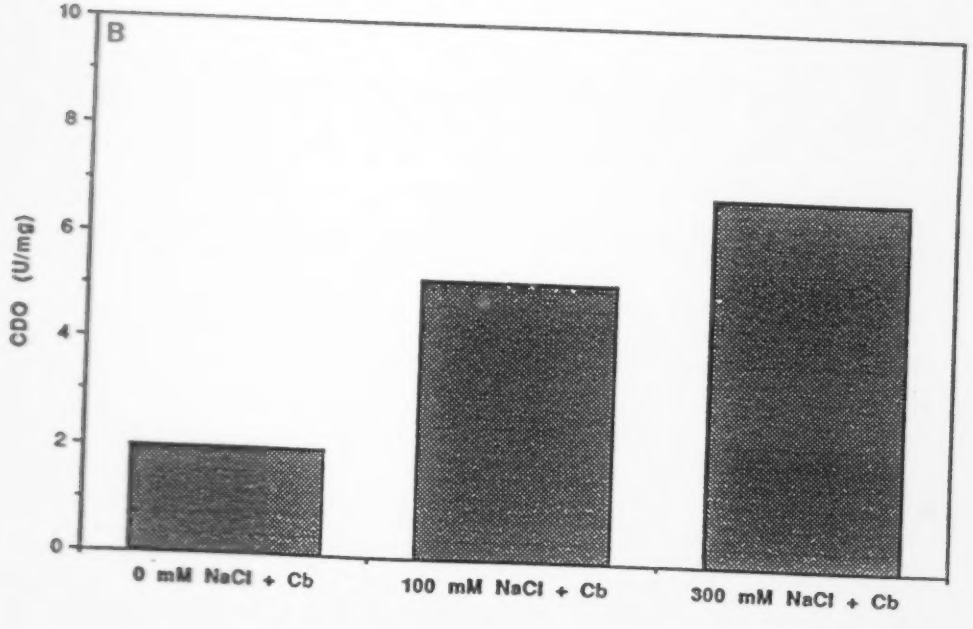
P. aeruginosa DMX 568/1 was grown on LB agar plates (with or without 300 μg/mL carbenicillin) containing 0, 100 or 300 mM NaCl. The resultant overnight growth was assayed for CDO reporter gene activity (as described in Materials and Methods section II.7.1, except that the cells were scraped off the plates) and the results are shown in Figure 17. In general, increasing the sodium chloride concentration resulted in an increase in CDO specific activity whether carbenicillin was present or not. The presence of carbenicillin appeared to decrease CDO activity compared to that observed for cells grown in the absence of carbenicillin.

III.4.2 Effect of culture conditions on growth and *algD* transcription in batch culture

Pseudomonas aeruginosa DMX 568/1 was grown in batch culture as described in Material and Methods (II.3). For each of the batch culture experiments, a range of NaCl concentrations from 0 to 600 mM was used. Culture conditions were varied as discussed below to investigate their effect on growth and catechol 2,3 dioxygenase reporter gene activity.

FIGURE 17. Effect of sodium chloride concentration on catechol 2,3 dioxygenase (CDO) activity of *Pseudomonas aeruginosa* DMX 568/1 grown on solid media (A) devoid of carbenicillin (cb) and (B) in the presence of carbenicillin (cb). The concentration of carbenicillin used was 300 μg/mL. One unit of CDO is defined as the amount of enzyme oxidizing 1 μmol of catechol per minute at room temperature.


A
10
8
6
4
2
0
CDO (U/mg)
0 mM NaCl - Cb
100 mM NaCl - Cb
300 mM NaCl - Cb

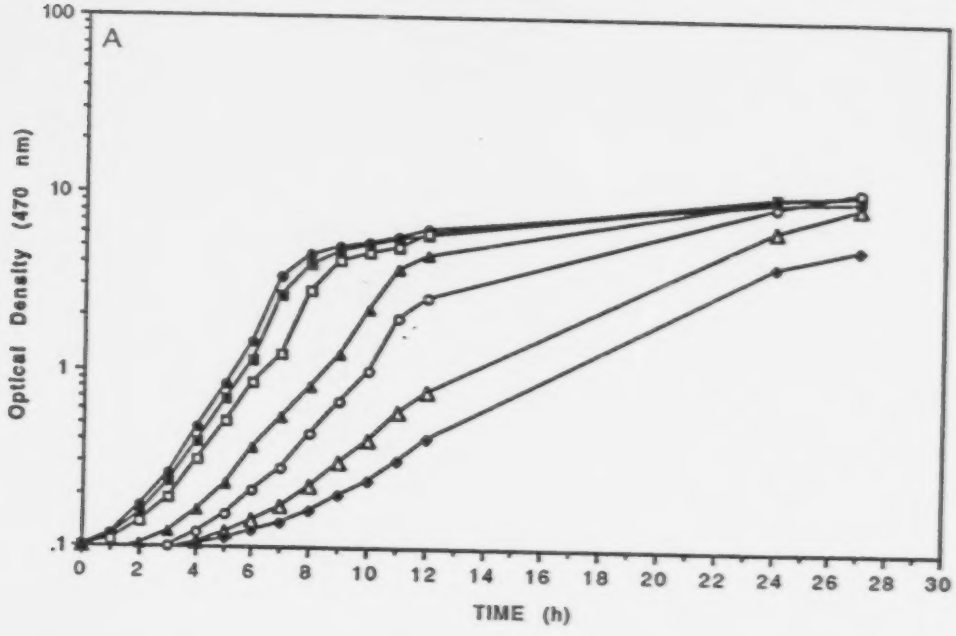

B
10
8
6
4
2
0
CDO (U/mg)
0 mM NaCl + Cb
100 mM NaCl + Cb
300 mM NaCl + Cb

III.4.2.2 Growth in Luria-Bertani medium devoid of carbenicillin

The growth curves for *Pseudomonas aeruginosa* DMX 568/1 grown in LB broth with increasing concentrations of NaCl at room temperature, 37°C and 39.5°C are shown in Figures 18A, 19A and 20A respectively. As expected, growth rates (as determined by the slopes of exponential curves) were faster at the warmer temperatures (37°C and 39.5°C) and slower at room temperature. At both room temperature (Fig. 18A), and at 37°C (Fig. 19A), the growth rates for cells grown in 0 and 100 mM NaCl were about the same. When the temperature was raised to 39.5°C (Fig. 20A), the growth rate was not affected by concentrations of NaCl up to and including 200 mM. Growth rates, for cells at all temperatures, started to drop off when the cells were grown in greater concentrations of NaCl. For all three temperatures, the slowest growth was observed for cells grown in 600 mM NaCl.

The CDO activity was also measured throughout growth for the various sodium chloride concentrations and is shown in Figures 18B, 19B and 20B. The activity of catechol 2,3 dioxygenase was not detected until the onset of stationary phase for cultures grown at room temperature (Fig. 18B), 37°C (Fig. 19A), and 39.5°C (Fig. 20A). Overall higher activity was observed at 37°C (Fig. 19B) compared with room temperature and 39.5°C. Cells grown at room temperature had detectable, but very low, CDO activity.

FIGURE 18. Effect of sodium chloride concentration on (A) growth and (B) catechol 2,3 dioxygenase (CDO) activity of *Pseudomonas aeruginosa* DMX 568/1 grown at room temperature in shake flask cultures of LB medium. One unit of CDO is defined as the amount of enzyme oxidizing 1 μmol of catechol per minute at room temperature. Symbols: 0 mM NaCl (●); 100 mM NaCl (■); 200 mM NaCl (□); 300 mM NaCl (▲); 400 mM NaCl (○); 500 mM NaCl (△); 600 mM NaCl (◆).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10
1
.1
Optical Density (470 nm)
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TIME (h)

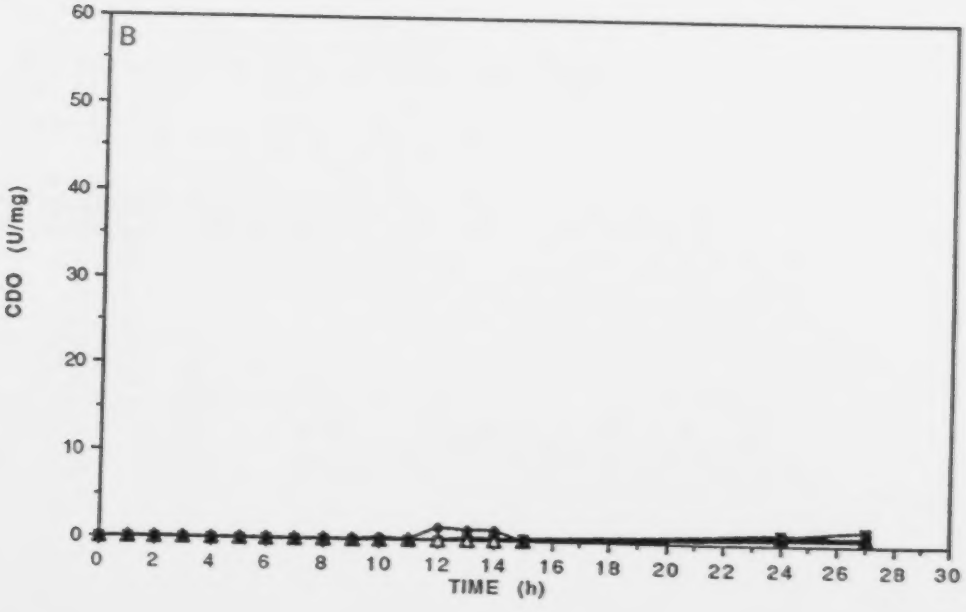
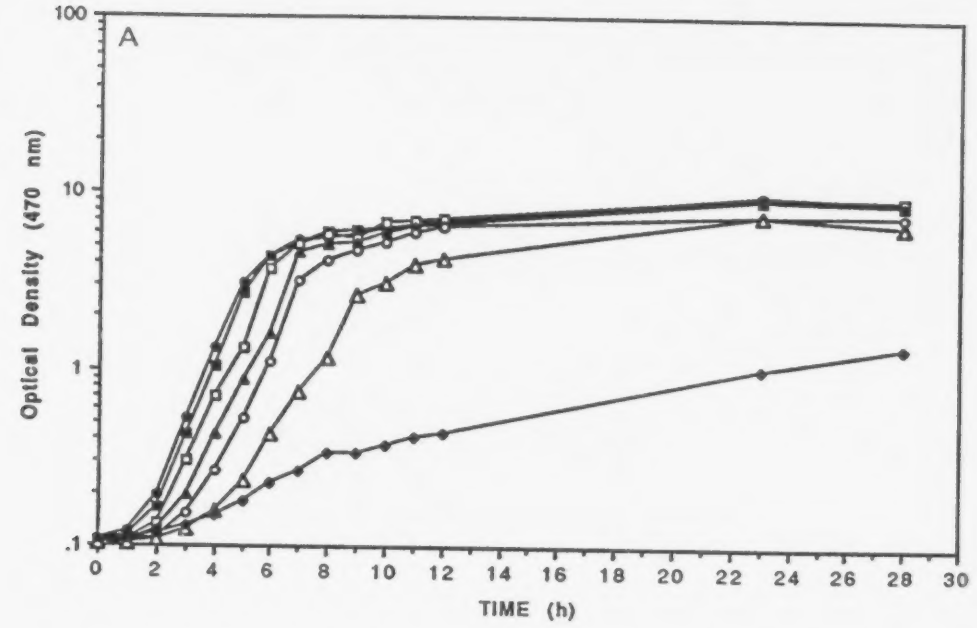

B
60
50
40
30
20
10
0
CDO (U/mg)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (h)

FIGURE 19. Effect of sodium chloride concentration on (A) growth and (B) catechol 2,3 dioxygenase (CDO) activity of *Pseudomonas aeruginosa* DMX 568/1 grown at 37°C in shake flask cultures of LB medium. One unit of CDO is defined as the amount of enzyme oxidizing 1 μmol of catechol per minute at room temperature. Symbols: 0 mM NaCl (●); 100 mM NaCl (■); 200 mM NaCl (□); 300 mM NaCl (▲); 400 mM NaCl (○); 500 mM NaCl (△); 600 mM NaCl (◆).


A
Optical Density (470 nm)
100
10
1
.1
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
TIME (h)

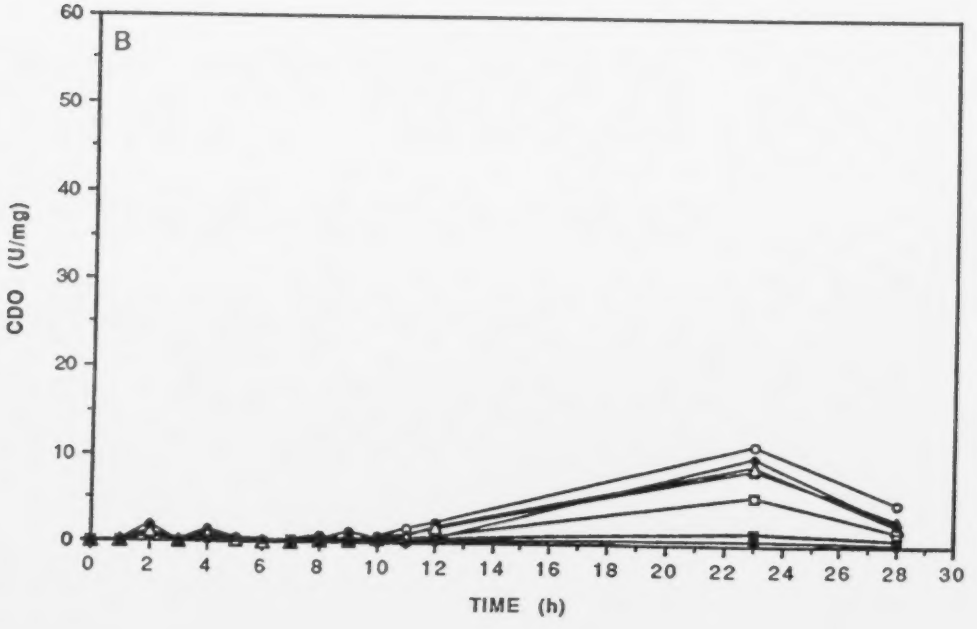
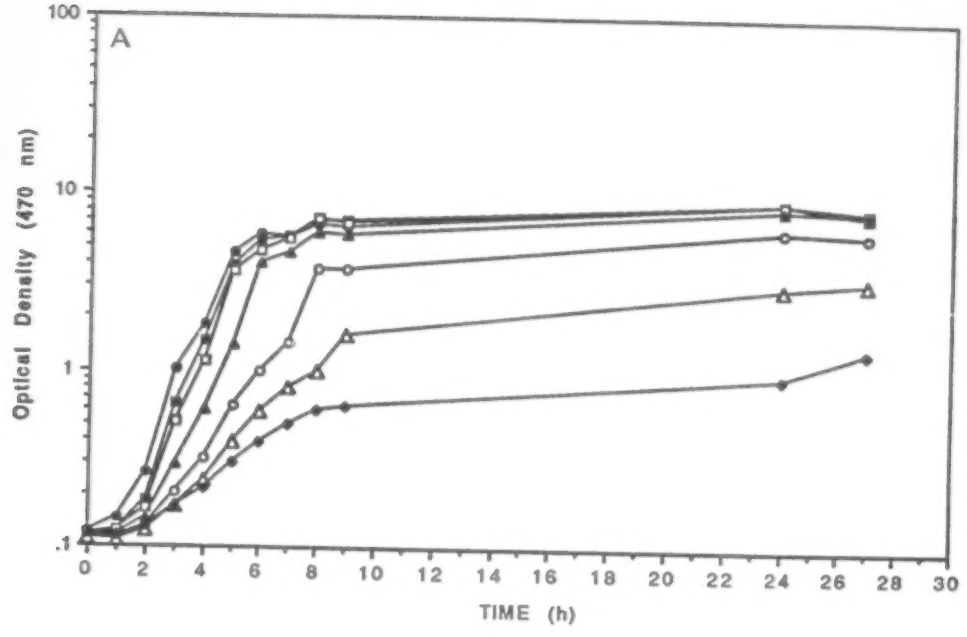
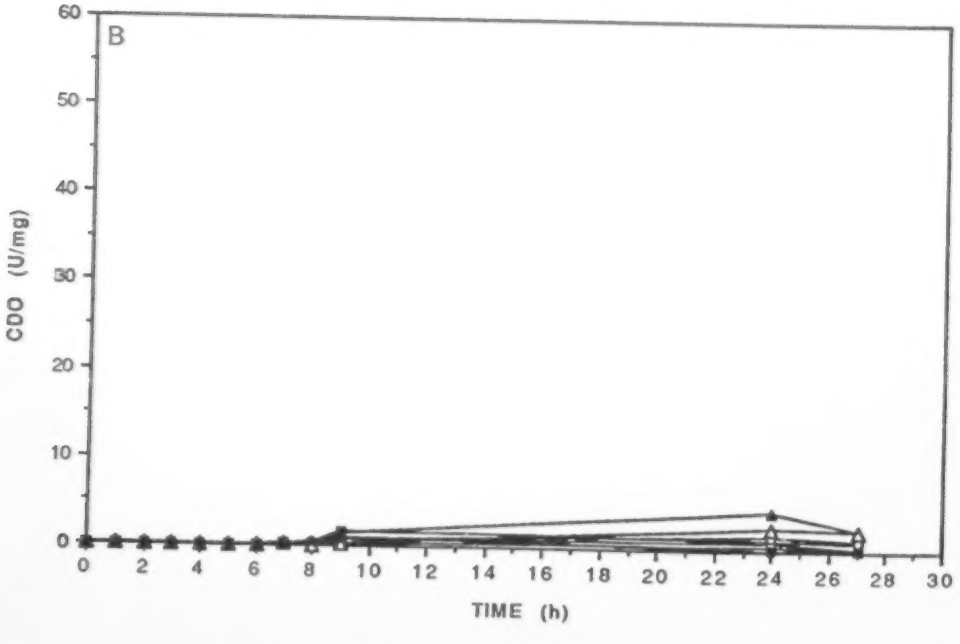

B
CDO (U/mg)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
TIME (h)

FIGURE 20. Effect of sodium chloride concentration on (A) growth and (B) catechol 2,3 dioxygenase (CDO) activity of *Pseudomonas aeruginosa* DMX 568/1 grown at 39.5°C in shake flask cultures of LB medium. One unit of CDO is defined as the amount of enzyme oxidizing 1 μmol of catechol per minute at room temperature. Symbols: 0 mM NaCl (●); 100 mM NaCl (■); 200 mM NaCl (□); 300 mM NaCl (▲); 400 mM NaCl (○); 500 mM NaCl (△); 600 mM NaCl (◆).


A
Optical Density (470 nm)
100
10
1
.1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (h)

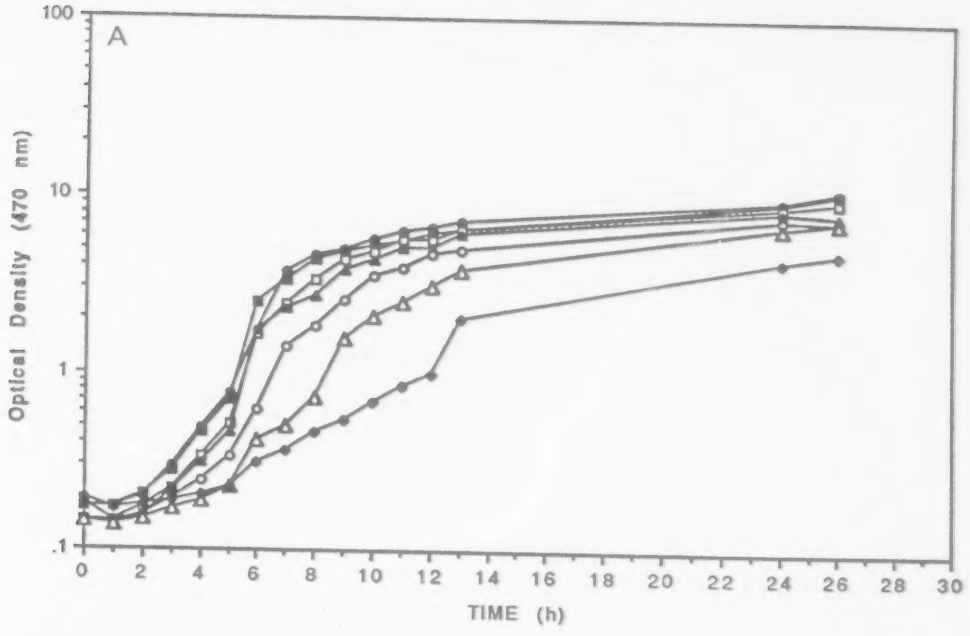

B
CDO (U/mg)
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (h)

III.4.2.1 Growth in Luria-Bertani medium containing carbenicillin

The growth curves for *Pseudomonas aeruginosa* DMX 568/1 grown in LB broth containing 300 μg/mL carbenicillin with increasing concentrations of NaCl at room temperature, 37°C and 39.5°C are shown in Figures 21A, 22A and 23A respectively. The growth rates observed for cells grown in 0, 100, and 200 mM NaCl are about the same for each of the three temperatures. Growth rates started to drop off when the cells were grown in greater than 300 mM NaCl. Cells grown at 37°C were most affected by NaCl concentration. The effect of salt on growth is minimal at room temperature. For all three temperatures, the slowest growth was observed for cells grown in 600 mM NaCl.

The CDO activity was measured throughout growth for the various sodium chloride concentrations and is shown in Figures 21B, 22B and 23B. Catechol 2,3 dioxygenase activity fluctuates during logarithmic growth but appears to be relatively constant in stationary phase, especially for those cells grown at 37°C (Fig. 22B). In general, a higher CDO activity is observed at 37°C compared to the lower and higher temperatures. In particular, a higher CDO activity is observed in cultures grown in the absence of NaCl at 37°C (Fig. 22A). Increased osmolarity does not produce a definite trend with respect to CDO activity.

FIGURE 21. Effect of sodium chloride concentration on (A) growth and (B) catechol 2,3 dioxygenase (CDO) activity of *Pseudomonas aeruginosa* DMX 568/1 grown at room temperature in shake flask cultures of LB medium containing 300 μg/mL carbenicillin. One unit of CDO is defined as the amount of enzyme oxidizing 1 μmol of catechol per minute at room temperature. Symbols: 0 mM NaCl (●); 100 mM NaCl (■); 200 mM NaCl (□); 300 mM NaCl (▲); 400 mM NaCl (○); 500 mM NaCl (△); 600 mM NaCl (◆).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10
1
.1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (h)

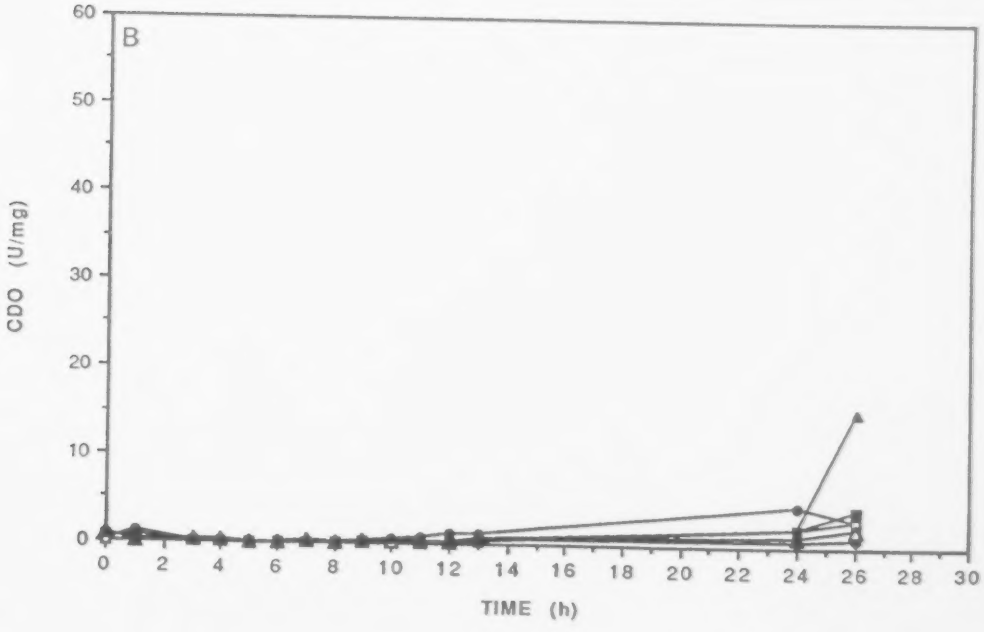
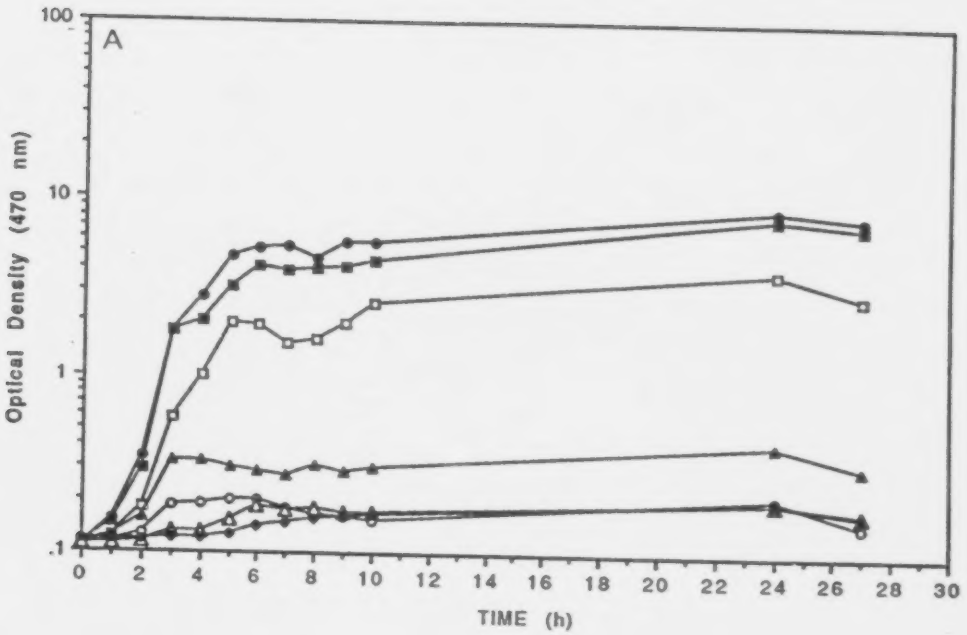

B
CDO (U/mg)
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (h)

FIGURE 22. Effect of sodium chloride concentration on (A) growth and (B) catechol 2,3 dioxygenase (CDO) activity of *Pseudomonas aeruginosa* DMX 568/1 grown at 37°C in shake flask cultures of LB medium containing 300 μg/mL carbenicillin. One unit of CDO is defined as the amount of enzyme oxidizing 1 μmol of catechol per minute at room temperature. Symbols: 0 mM NaCl (●); 100 mM NaCl (■); 200 mM NaCl (□); 300 mM NaCl (▲); 400 mM NaCl (○); 500 mM NaCl (△); 600 mM NaCl (◆).


A
Optical Density (470 nm)
100
10
1
.1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (h)

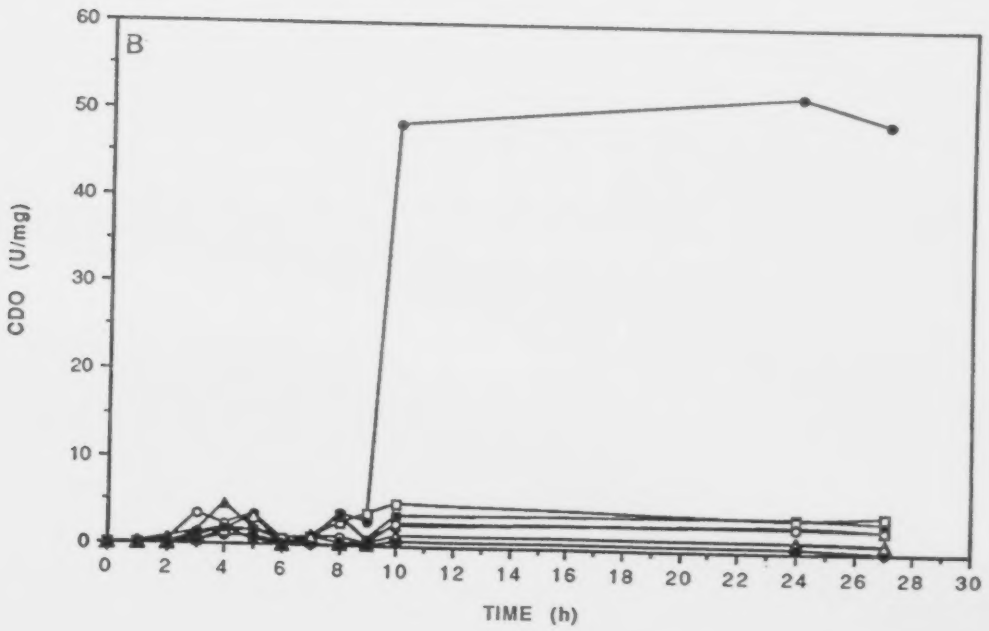
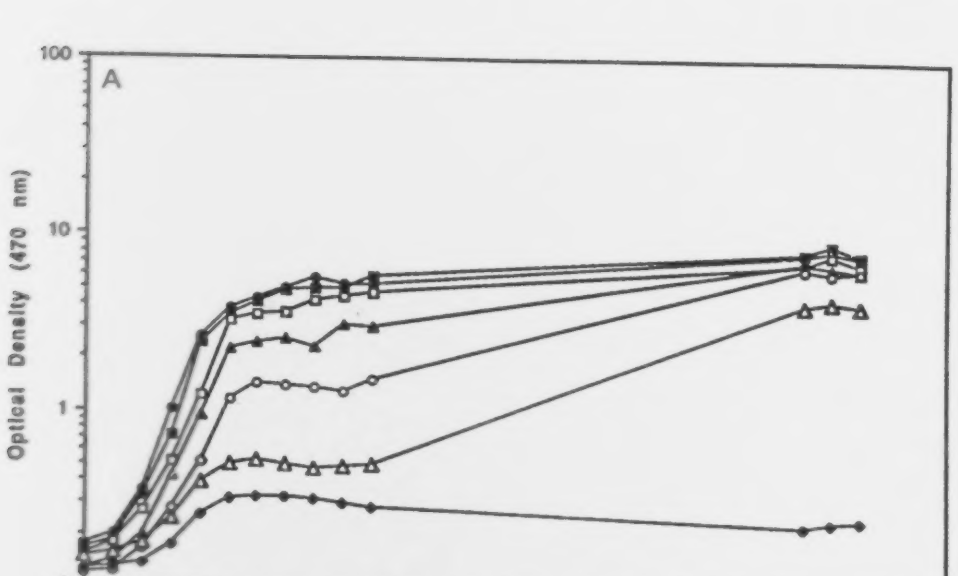
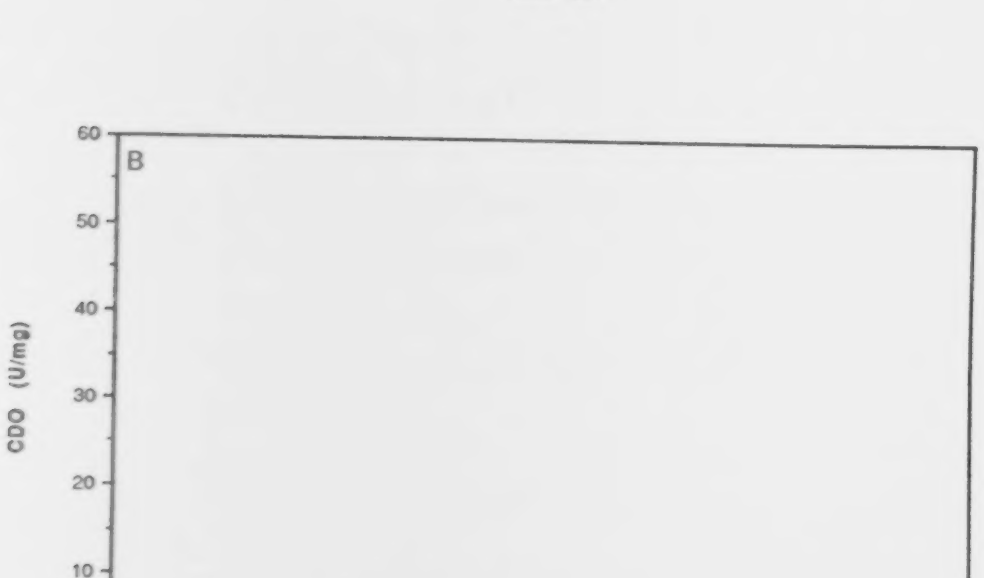

B
CDO (U/mg)
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (h)

FIGURE 23. Effect of sodium chloride concentration on (A) growth and (B) catechol 2,3 dioxygenase (CDO) activity of *Pseudomonas aeruginosa* DMX 568/1 grown at 39.5°C in shake flask cultures of LB medium containing 300 μg/mL carbenicillin. One unit of CDO is defined as the amount of enzyme oxidizing 1 μmol of catechol per minute at room temperature. Symbols: 0 mM NaCl (●); 100 mM NaCl (■); 200 mM NaCl (□); 300 mM NaCl (▲); 400 mM NaCl (○); 500 mM NaCl (△); 600 mM NaCl (◆).


A
Optical Density (470 nm)
100
10
1
.1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (h)


B
CDO (U/mg)
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (h)

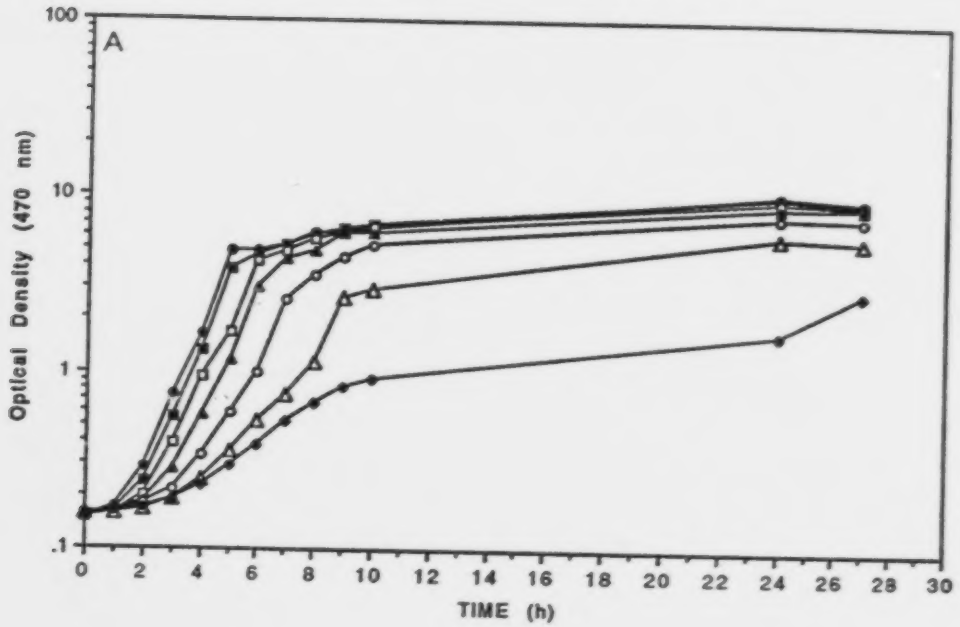
III.4.2.3 Growth in Luria-Bertani medium containing 2,2'-dipyridyl

Iron is a nutritional requirement for the growth of most pathogens since it is required for many metabolic processes. In the body, the concentration of free iron is very low. In the following experiment, the iron chelator 2,2'-dipyridyl was added to sequester free iron in the medium so that the effect of iron restriction on *algD* transcription could be determined.

The growth curves for *P. aeruginosa* DMX 568/1 grown in LB broth containing 25 μg/mL 2,2'-dipyridyl with increasing salt concentrations are illustrated in Figures 24A (37°C), 25A (39.5°C). For both temperatures, growth rate begins to decline when the sodium chloride concentrations are increased beyond 200 mM. Temperature has little effect on growth in the presence of 2,2'-dipyridyl.

CDO activity was measured throughout growth (Figs. 24B and 25B). The pattern of expression of *algD* as measured by CDO activity differs for the two temperatures Overall, the CDO activity observed in the presence of 2,2'-dipyridyl appears to be much lower at 37°C than at 39.5°C throughout growth. In cultures grown at 39.5°C, expression appears to be quite erratic prior to stationary phase, but no definite trend emerges on the effect of NaCl concentration on CDO activity.

FIGURE 24. Effect of sodium chloride concentration on (A) growth and (B) catechol 2,3 dioxygenase (CDO) activity of *Pseudomonas aeruginosa* DMX 568/1 grown at 37°C in shake flask cultures of LB medium containing 2,2'-dipyridyl (25 μg/mL). One unit of CDO is defined as the amount of enzyme oxidizing 1 μmol of catechol per minute at room temperature. Symbols: 0 mM NaCl (●); 100 mM NaCl (■); 200 mM NaCl (□); 300 mM NaCl (▲); 400 mM NaCl (○); 500 mM NaCl (△); 600 mM NaCl (◆).


A
Optical Density (470 nm)
100
10
1
.1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (h)

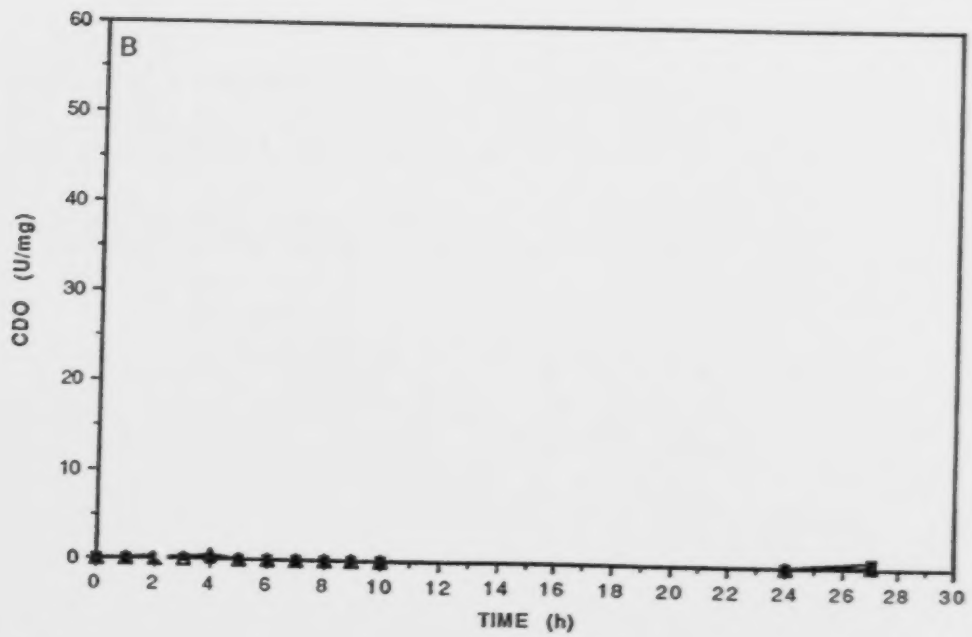
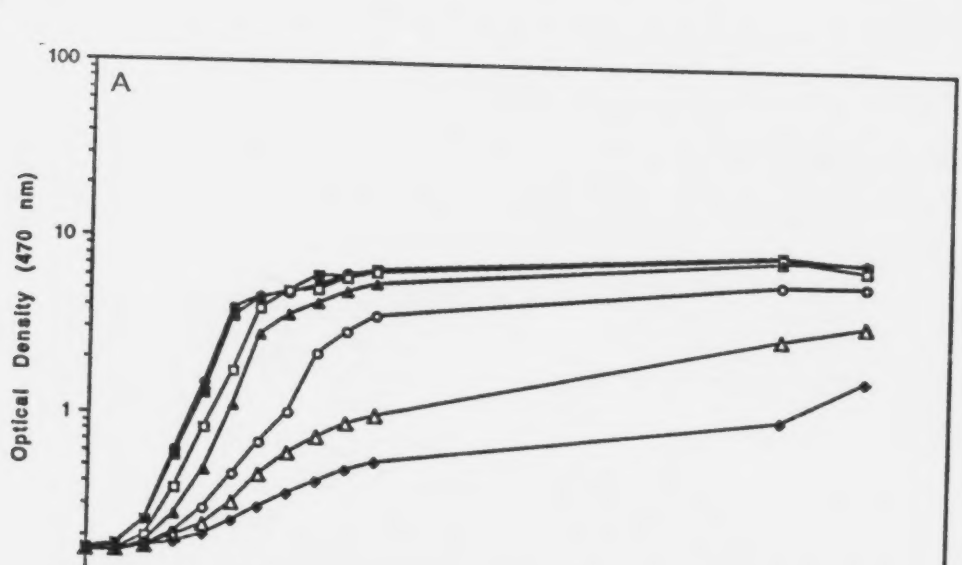
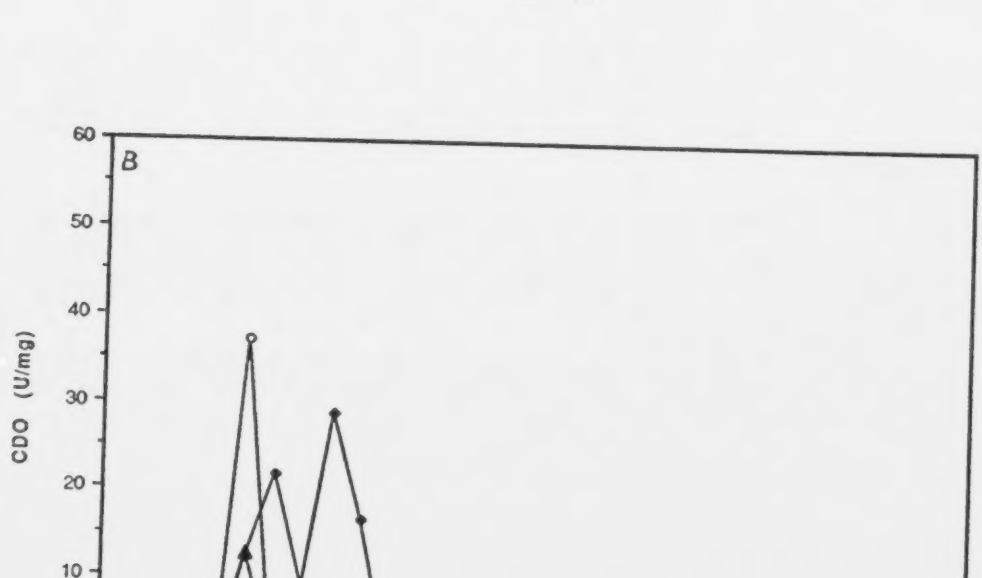

B
CDO (U/mg)
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (h)

FIGURE 25. Effect of sodium chloride concentration on (A) growth and (B) catechol 2,3 dioxygenase (CDO) activity of *Pseudomonas aeruginosa* DMX 568/1 grown at 39.5°C in shake flask cultures of LB medium containing 2,2'-dipyridyl (25 μg/mL). One unit of CDO is defined as the amount of enzyme oxidizing 1 μmol of catechol per minute at room temperature. Symbols: 0 mM NaCl (●); 100 mM NaCl (■); 200 mM NaCl (□); 300 mM NaCl (▲); 400 mM NaCl (○); 500 mM NaCl (△); 600 mM NaCl (◆).


A
Optical Density (470 nm)
100
10
1
.1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (h)


B
CDO (U/mg)
60
50
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TIME (h)

III.4.2.4 Growth in bovine serum containing carbenicillin

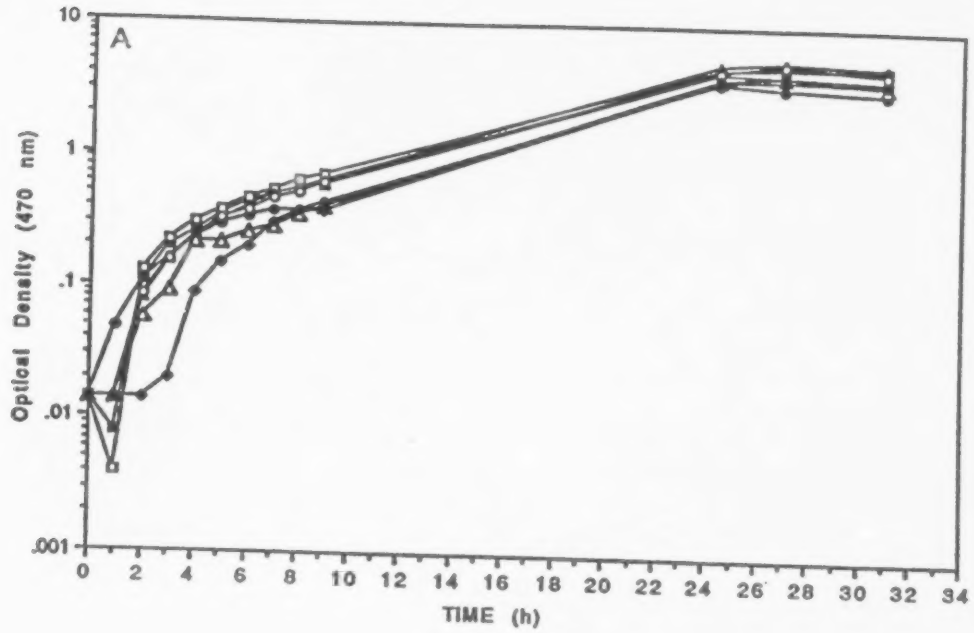
Infections are often accompanied by an elevated temperature. It has been previously shown that some organisms have an increased resistance to killing by whole blood at lower rather than higher temperature. It was therefore of interest to study the effect of serum and temperature on *algD* transcription.

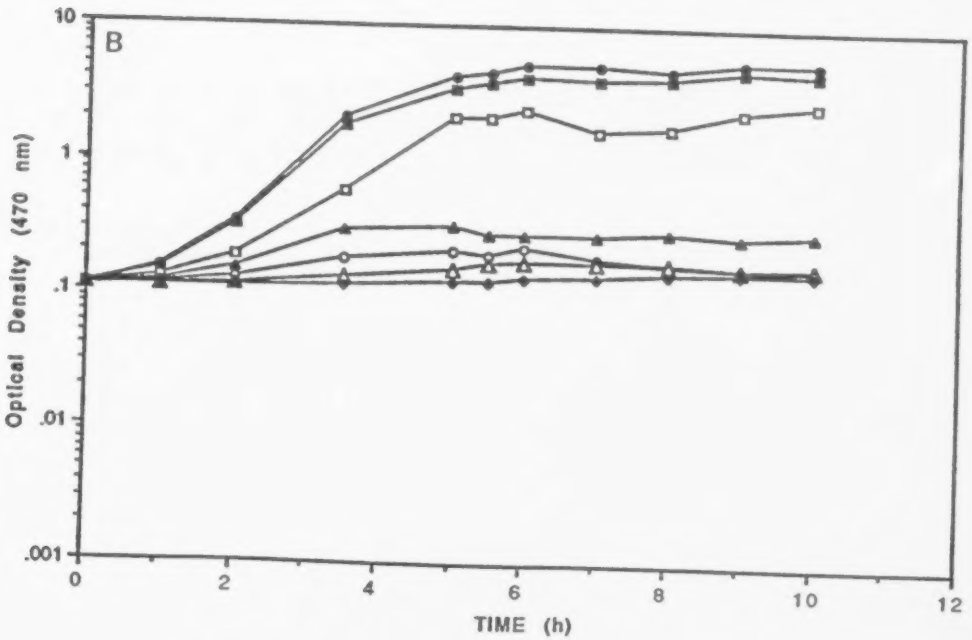
The growth curves for *P. aeruginosa* DMX 568/1 grown in bovine serum (plus 300 μg/mL carbenicillin) containing increasing concentrations of NaCl at 37°C are shown in Figure 26A. As can be seen from the Figure, the concentration of sodium chloride has almost no effect on growth at 37°C .

The growth curves for *P. aeruginosa* DMX 568/1 grown in bovine serum (plus 300 μg/mL carbenicillin) containing increasing concentrations of NaCl at 39.5°C are shown in Figure 26B. The results observed for this temperature are quite different from those observed at 37°C. AT 39.5°C, growth in serum is clearly inhibited by increasing concentrations of NaCl. These results therefore suggest that at elevated temperature, serum killing is more pronounced under conditions of high osmolarity.

Catechol 2,3 dioxygenase activity was undetectable in cells grown in bovine serum (containing 300 μg/mL carbenicillin) at all concentrations of NaCl used in this experiment. A lack of activity could not be attributed to the inoculum since a sample of the LB-grown inoculum displayed CDO activity when assayed. To further investigate this loss of activity, *P.*

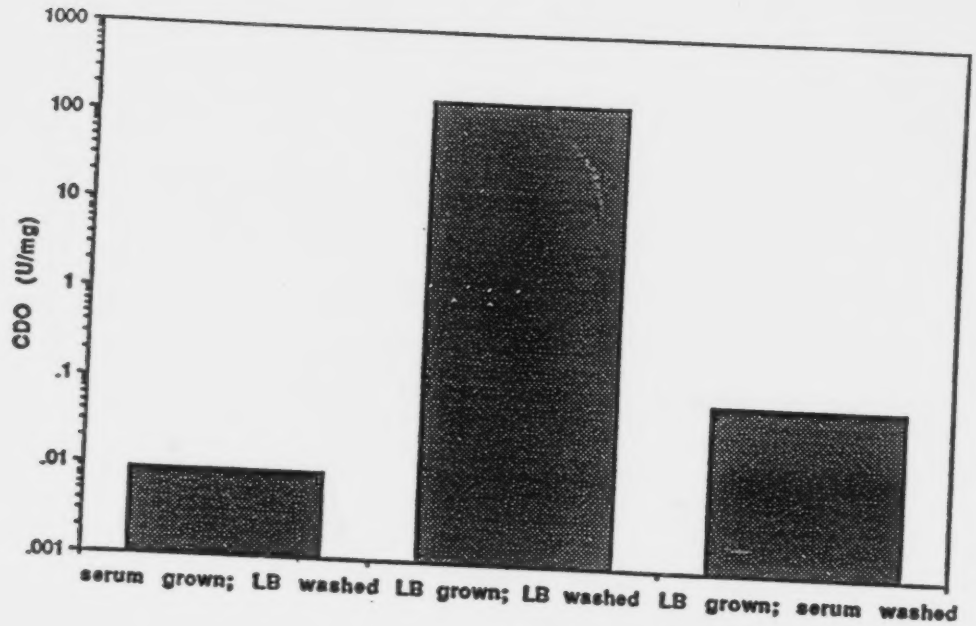
FIGURE 26. Effect of sodium chloride concentration on the growth of *Pseudomonas aeruginosa* DMX 568/1 in bovine serum containing 300 μg/mL carbenicillin in shake flask cultures at (A) 37°C and (B) 39.5°C. Symbols: 0 mM NaCl (●); 100 mM NaCl (■); 200 mM NaCl (□); 300 mM NaCl (▲); 400 mM NaCl (○); 500 mM NaCl (△); 600 mM NaCl (◆).


A
Optical Density (470 nm)
10
1
.1
.01
.001
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
TIME (h)


B
Optical Density (470 nm)
10
1
.1
.01
.001
0 2 4 6 8 10 12
TIME (h)

aeruginosa DMX 568/1 was grown in LB medium containing 300 μg/mL carbenicillin in batch culture as described in Materials and Methods (II.3). Growth was allowed to proceed to stationary phase. Previous growth experiments performed in LB always indicated measurable activity in stationary phase. Pellets of both stationary phase cultures were washed with either serum (plus carbenicillin) or LB (plus carbenicillin) and then assayed for activity. The results are shown in Figure 27. When the serum-grown cells were washed with serum, no detectable CDO activity was observed. When serum-grown cells were washed with LB, a small amount of activity was detectable. In contrast, LB-grown, LB-washed cells showed activity consistent with other batch experiments carried out under similar growth conditions. When serum was used to wash LB-grown cells, activity decreased dramatically. The reason for this difference in activity was not investigated further. It is possible that a serum component either interferes with the assay or perhaps degrades or inhibits catechol 2,3 dioxygenase activity.

FIGURE 27. Effect of bovine serum on the catechol 2,3 dioxygenase (CDO) activity of *Pseudomonas aeruginosa* DMX 568/1 grown in shake flask cultures. One unit of CDO is defined as the amount of enzyme oxidizing 1 μmol of catechol per minute at room temperature.


1000
100
10
1
.1
.01
.001
CDO (U/mg)
serum grown; LB washed
LB grown; LB washed
LB grown; serum washed

IV. DISCUSSION

Growth environment and genetic composition determines the pathogenic behaviour of bacteria. *In vivo*, the cell responds to environmental signals. This may involve altering the patterns of gene expression (Brown and Williams, 1985; Miller *et al.*, 1989, Deretic *et al.*, 1987a; 1987b; 1990; DeVault *et al.*, 1989) as well as altering the susceptibility to antibiotics (Anwar and Costerton, 1990). The degree of susceptibility of bacterial pathogens to any group of antibiotics depends on the conditions used for cell cultivation. It is important that these parameters be adequately defined. Growth parameters known to affect the physiological functions of bacterial cells *in vivo* are vital to experimental design, especially for the evaluation of antibiotic efficacies (Anwar *et al.*, 1990)

The colonization of medical devices poses a serious threat to patients undergoing invasive surgery for prosthetic implants. The ability of *Pseudomonas aeruginosa* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) to colonize silicone tubing, a material used in the manufacture of catheters, was investigated. Both planktonic (free-living) and biofilm (sessile) bacteria were exposed to a variety of environmental stresses ranging from antibiotic exposure to conditions of high osmolarity. The latter has been implicated in the mucoid phenotype observed with *P. aeruginosa* colonizing the cystic fibrosis (CF)-affected lung.

S. aureus is commonly associated with infections in patients receiving peritoneal catheters (Vas, 1983; 1989). ß-Lactam and aminoglycoside antibiotics are routinely used in combination for the treatment of such biofilm-associated infections (Anwar and Costerton, 1990; Vas, 1989), and are incorporated into the peritoneal dialysis fluid (Vas, 1983; 1989). The

concentrations of antibiotics described in this study (section III.1.1) are achievable in the dialysis fluid used in the treatment of peritoneal infections. Planktonic cells of *S. aureus* were found to be sensitive to tobramycin (5 μg/mL) and cephalexin (100 μg/mL). A combination of tobramycin and cephalexin at these concentrations enhanced the rate of killing of the planktonic cells. Although young *S. aureus* biofilms are slightly more resistant than planktonic cells to tobramycin and cephalexin, they can be eliminated with a combination of these antibiotics (5 μg/mL tobramycin plus 100 μg/mL cephalexin). In contrast, although higher concentrations of tobramycin and cephalexin used in combination (10 μg/mL tobramycin plus 100 μg/mL cephalexin) enhances the rate of killing of aged biofilm cells they were not sufficient for eradication unless toxic dosages (levels considered toxic to the patient) of these antibiotics were employed.

Antibiotics play a profound role in the control of bacterial infections. The concentrations of antibiotics *in vivo* are dependent on the rate of clearance of the antibiotics from the body. By administering antibiotics directly into the chemostat system, it becomes possible to study the effects of antibiotic clearance on bacterial cells. Planktonic and young biofilm cells of the 2-day-old chemostat culture of *S. aureus* were effectively eradicated by the loading (70 μg/mL tobramycin and 500 μg/mL cephalexin) and maintenance (62.5 μg/mL tobramycin and 250 μg/mL cephalexin) doses of tobramycin and cephalexin used (Fig. 7). Regrowth did not occur after antibiotic treatment was discontinued on day 7. When antibiotic treatment was initiated on day 21 rather than day 2, a rapid decrease in the cell viability was still observed when the planktonic and old biofilm cells were exposed to the loading dose of tobramycin and cephalexin (Fig. 8), however complete eradication was not

observed even when the antibiotic exposure was continued for an extra six days (Fig. 8). Regrowth of the organism occurred when antibiotic exposure was terminated. [The regrowth that was observed for the 21-day old planktonic cultures was probably due to the sloughing off of old biofilm cells (Figs. 7 and 8) due to turbulence created by air bubbles supplied to the system.] The loading and maintenance doses of tobramycin and cephalexin used were not sufficient to completely eradicate the sessile bacteria and higher levels were not tested because they would be too toxic to the patient if used in a clinical setting.

P. aeruginosa is an opportunistic pathogen involved in medical device-associated infections. The effect of dilution rate on biofilm formation, as well as expression of iron-regulated outer-membrane proteins was investigated. Changes in the dilution rate dramatically affected the populations of planktonic and biofilm cells of *P. aeruginosa* (Figs. 9, 10A, 10B, 11A, 11B, and 12). A rapid decline of the planktonic population resulted from increasing the dilution rate from 0.2 to 1.0 h^{-1} (Figs. 9 and 12). This is generally observed when dilution rate is increased in a chemostat since the maximum growth rate (μ_{max}) of the organism is lower than the dilution rate used. That is, the cells could not grow faster than the flow of the medium into the chemostat and the planktonic cells were washed out. The number of planktonic cells however, did not decrease to zero, and a new equilibrium state was established when the dilution rate was increased to 1.0 h^{-1}. This is likely due to the release of loosely bound biofilm cells from the surfaces of the silicone tubing.

From the dilution rate studies, it was observed that the number of biofilm cells colonizing the surface of the silicone tubing increases as the dilution rate is increased. This holds true for both mucoid and non-mucoid *P.*

aeruginosa (Figs. 10A and 10B). Since there are fewer planktonic cells competing for limited nutrients as the result of the decrease in the population of the planktonic cells when the dilution rate is increased, the biofilm cells presumably have greater access to the limited nutrients thus leading to an increase in the number of biofilm cells colonizing the silicone tubing. The number of biofilm cells of the mucoid strain of *P. aeruginosa* was consistently higher than the non-mucoid strain. (Figs. 10A and 10B) suggesting that the production of alginate by the mucoid *P. aeruginosa* 492a may promote biofilm formation. However, since alginate production was not quantitated, conclusive data is not available. Also, differences observed with the growth rate of strains could arise from genotype differences; these differences could be investigated further by the use of isogenic strains

Since restriction of available iron is an important nonspecific host defense against microorganisms (Anwar *et al.,* 1984; Brown *et al.,* 1984; Griffiths *et al.,* 1983), iron-regulated outer-membrane proteins (IROMPs) are expressed by pathogenic bacteria *in vivo.* In this study, the expression of IROMPs was strongly induced in both planktonic and biofilm cells at dilution rates of 0.05 and 0.2 h^{-1} (low dilution rates). The intensity of the IROMPs was sharply reduced when the dilution rate was increased (Plate 3). The IROMPs were barely detectable in the outer membrane (OM) preparations of biofilm cells cultivated at the dilution rate of 1.0 h^{-1}. Because of the presence of a relatively large planktonic population in the chemostat, both planktonic and biofilm cells of *P. aeruginosa* probably compete for the trace amount of iron present in the growth environment at low dilution rates (0.05 and 0.2 h^{-1}). It is well documented that cells switch on the high affinity iron uptake system to assist in the acquisition of iron and this involves expression of IROMPs which

function as iron-siderophore complexes (Griffiths, 1983). More iron was made available when the dilution rate was increased resulting from an increase in the flow of medium to the chemostat as well as a rapid reduction in the population of planktonic cells, hence there would be no need for the biofilm cells to switch on the high affinity iron uptake system. This is what was observed in these experiments, where the expression of IROMPs did not increase when the dilution rate was increased.

Antibiotics with activity against *Pseudomonas aeruginosa* play a critical role in reducing mortality in CF patients (Hoiby *et al.*, 1982). It is believed that the biofilm mode of growth may be responsible for the persistence of this organism in the CF-affected lung (Costerton *et al.*, 1983; Lam *et al.*, 1984; Nickel *et al.*, 1985b). Chemostat-controlled doses of piperacillin (ß-lactam antibiotic) and tobramycin (aminoglycoside) can be used to effectively eliminate the planktonic and young biofilm cells of mucoid *P. aeruginosa* M 579 (Fig. 15). Old biofilm cells of *P. aeruginosa* M 579 did not respond to the chemostat-controlled doses of piperacillin and tobramycin, even when the concentration of piperacillin was increased to 500 μg/mL (Fig. 16). The percentage of survival remained in the range of 20 to 40% during the course of antibiotic therapy. The population of the old biofilm cells increased back to 100% after the termination of antibiotic treatment on day seven. This confirmed earlier observations that old biofilm cells of *P. aeruginosa* M 579 were resistant to these antibiotics. These results suggest that the establishment of aging biofilms is a possible mechanism of bacterial persistence despite antimicrobial therapy employed in medical device-associated infections. This mechanism is likely to be very complex and may

involve changes in the permeability barrier, the production of bacterial enzymes, and the molecular targets of antibiotics.

Since the mucoid phenotype of *P. aeruginosa* is an important virulence factor in cystic fibrosis, studies were undertaken to gain a greater understanding of environmental factors affecting alginate formation. The expression of *algD* was studied using solid medium and a variety of conditions in batch culture (due to time constraints, continuous culture studies were not completed). The transcription of *algD* was assessed using a transcriptional fusion of the *algD* promoter region to the promoterless *xylE* reporter gene (encodes for catechol 2,3 dioxygenase (CDO) which cleaves the colorless catechol to 2-hydroxymuconic semialdehyde, a yellow compound). A simplistic picture of the effect of salt concentration on *algD* expression is observed when *P. aeruginosa* DMX 568/1 (the strain containing the *algD/xylE* fusion) is grown on solid media. An increase in NaCl concentration results in an increased CDO activity. This trend is in agreement with observations of other researchers (Berry *et al.*, 1989). *P. aeruginosa* DMX 568/1 yielded less overall CDO activity than strains reported in the literature, this may be attributed to strain differences. In the presence of carbenicillin, CDO activity decreases but the trend remains that an increase in osmolyte results in an increased *algD* expression on solid media. [Carbenicillin was added to the media since it was initially thought to be required for stable maintenance of the chromosomal *algD/xylE* fusion.] Previous workers (DeVault *et al.*, 1987) showed that when KCl was substituted for NaCl at concentrations giving equal ionic strength (conductance), similar results were obtained as for NaCl, indicating that osmolarity and not NaCl is responsible, however the effects of KCl were not addressed in the studies presented here. Berry *et al.* (1989)

found that increased activation of *algD* at high osmolarity was not sufficient to induce alginate synthesis in non-mucoid strains and suggested that other environmental factors are involved in full activation of the alginate pathway.

In addition to the studies in which solid medium was employed, the effect of NaCl concentration in liquid batch culture was also addressed. In this case, the results were quite different from those obtained on solid media. Depending on the culture conditions, such as addition of carbenicillin or 2,2'-dipyridyl (to establish conditions of iron limitation), and varying growth temperature, a wide range of results were observed. In fact, expression of *algD* did not appear to be initiated at any particular point in the growth cycle, nor was there any obvious effect of NaCl concentration on CDO activity. There was considerable variability with respect to the effect of NaCl under the various culture conditions (Figs. 18B to 25B) and no obvious trends emerged. Although osmolarity affects growth rate, growth rate does not appear to have any distinct effect on CDO activity in these studies. A decrease in CDO activity did not follow any decrease in growth rate. Alves *et al.* (1991) found that alginate in the growth medium led to a significant decrease of specific growth rates in the *P. aeruginosa* strains they studied. The more alginate produced, the lower the specific growth rates. Alginate increases medium viscosity with a consequent decrease in oxygen transfer. Because of the observations of Alves *et al.*, it would have been useful in our studies to quantitate alginate. Such data would have indicated whether or not any increase in *algD* transcription (in *P. aeruginosa* DMX 568/1) resulted in a corresponding increase in alginate production. In the studies presented here, medium viscosity was observed with increased NaCl concentration, however as stated above the possibility that the increase in viscosity was due to alginate production was not

addressed. It is possible that viscosity is unrelated to alginate production since increased CDO activity did not correspond to the increase in viscosity. Cell lysis and subsequent release of DNA into the medium could also have contributed to medium viscosity.

Although some trends could be seen in the expression of *algD* in response to different environmental conditions, the work reported in this thesis is preliminary at best. Further experimental work is required before any definite conclusions can be drawn.

It is important to realize that a biofilm is a dynamic microcosm. New members are constantly being added while old ones die or are removed. In the case of infection, one has to consider phagocytes which engulf planktonic cells and some surface layer cells. These phagocytes work in concert with antibiotics to reduce the number of bacteria at the site of infection. Clinical symptoms may disappear as the body recognizes the neutralized surface of the biofilm. Infection can recur as embedded biofilm cells break their dormancy and as fresh nutrients become available once surrounding layers of cells are removed. Although *in vitro* studies cannot provide all of the information required to devise methods to control biofilm formation or methods to eradicate established biofilms, such studies do lead to an increased knowledge of the complex interactions occurring: whether or not this knowledge can be applied to the *in vivo* situation remains to be demonstrated.

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